Univerza v Ljubljani | Medicinska fakulteta
INŠTITUT ZA MIKROBIOLOGIJO IN IMUNOLOGIJO

ISAC
International Society for Advancement of Cytometry

Institute of Pharmacology and Experimental Toxicology

Slovenian Society for Flow Cytometry (SSC)

ISAC LEFT LJUBLJANA CYTOMETRY WORKSHOP

INTERNATIONAL SCIENTIFIC WORKSHOP

May 4-6, 2018

Zbornik prispevkov z recenzijo | Proceedings

ISAC LETF delavnica pretočne citometrije Ljubljana 2018 | ISAC LETF Ljubljana Cytometry Workshop

Mednarodna znanstvena delavnica | International scientific workshop, Ljubljana 4. do 6. maj 2018

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ISAC LETF delavnica pretočne citometrije Ljubljana 2018 | ISAC LEFT Ljubljana Cytometry Workshop

Prizorišče | Venue
Medicinska fakulteta,
Korytkova 2,
1000 Ljubljana

Datum |Dates
6th - 8th May, 2018

Soorganizatorji | Co-organizers
ISAC (International Society for the Advancement of Cytometry),
Slovensko združenje za pretočno citometrijo,
Inštitut za mikrobiologijo in imunologijo,
Inštitut za farmakologijo in eksperimentalno toksikologijo
Medicinske fakultete Univerze v Ljubljani

Organizacijski odbor simpozija | Members of the organization committee

Awtar Krishan (Chair of the Live Education Task Force of ISAC)
Zosia Maciorowski (Chair of the Education Committee of ISAC)
Tomas Kalina (Chair of CSAC and Euro Flow founding member)
Andreja Nataša Kopitar (University of Ljubljana, Faculty of Medicine)
Katerina Černe (University of Ljubljana, Faculty of Medicine)
Alojz Ihan (University of Ljubljana, Faculty of Medicine)

Znanstveno recenzentski odbor|Board of Scientific Reviewers:

Assoc. Prof. Andreja Nataša Kopitar, Ph.D.
Prof. Alojz Ihan, Ph.D., M.D.

Zahvala |Acknowledgement
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Program znanstvenega simpozija |Program of scientific symposium:

Vsebina zbornika | Proceedings Contents

Program	**ISAC LEFT LJUBLJANA CYTOMETRY WORKSHOP**
Zosia Maciorowski	**BASICS OF FLOW CYTOMETRY**
Tomas Kalina	**BASIC DATA ANALYSIS AND PRESENTATION**
Zosia Maciorowski	**BASIC MULTICOLOR FLOW CYTOMETRY - FLUOROCHROMES, SPILLOVER AND COMPENSATION**
Zosia Maciorowski	**MULTICOLOR PANEL DESIGN**
Alojz Ihan	**FCM DIAGNOSTICS AND FUNCTIONAL TESTS IN AUTOINFLAMMATORY DISEASES - CLINICAL CASES**
David Hedley & Sue Chow	**SURFACE AND INTRACELLULAR FLOW CYTOMETRY**
Andreas Spittler	**EXTRACELLULAR VESICLES BASICS - PITFALLS - ARTEFACTS**
Claudio Vallan	**DATA ANALYSIS**

ISAC LETF

Ljubljana Cytometry Workshop

2018

May 4 – 6, 2018
Ljubljana | Slovenia

WORKSHOP VENUE

Faculty of Medicine, University of Ljubljana
Korytkova 2, Ljubljana, Slovenia

Lectures during the whole course will take place at the Medical faculty, Medium lecture hall (Srednja predavalnica).

FACULTY MEMBERS & WET-LAB LEADERS

Sue Chow | University of Toronto and Princess Margaret Hospital, Toronto, Canada

David Hedley | University of Toronto and Princess Margaret Hospital, Toronto, Canada

Tomas Kalina | CLIP Cytometry, Department of Pediatric Hematology and Oncology, Charles University, Prague, Czech Republic

Zosia Maciorowski | Curie Institute, Paris, France

Hana Glier | FACS/Stem cell laboratory, Institute of Laboratory Medicine, Cantonal Hospital Aarau, Switzerland

Alojz Ihan | Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia

Matthias Schiemann | Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München, Germany

Andreas Spittler | Core Facility Flow Cytometry & Surgical Research Laboratories, Medical University of Vienna, Austria

Bill Teldford | Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

Claudio Vallan | FACS Laboratory, Department of Clinical Research, University of Bern, Switzerland

MEETING ROOMS / LABORATORIES

W1 **Room No. 1, 5th floor** | Bio-Rad, S3 sorter

W2 **Room No. 2, 5th floor** | Becton Dickinson, BD FACSCanto II cell sorter

W3 **Room No. 3, 5th floor** | Becton Dickinson, BD FACSLyric

W4 **Room No. 4, 5th floor** | Meeting room

W5 **Room No. 5, 4th floor** | Beckman Coulter, CytoFLEX

ML **Ground floor** | Medium lecture hall

Ground floor foyer: coffee breaks, lunches, registration & information desk

ATTENDEES GROUPS

A: Experimental

B: Basic

C: Clinical

D: Group combined

ACCOMPANYING PROGRAM

Saturday, May 5, 2018

Guided tour of Ljubljana | 18:00 meeting point at the registration desk

Social dinner | 20:00, Gostilna na gradu, Grajska planota 1, Ljubljana

SCHEDULE

Friday, May 4, 2018

ML **Medium lecture hall**

12:00 – 13:00	**Registration** (open during the whole workshop)
13:00 – 13:15	**WELCOME**
13:15 – 14:00	**Basics of flow cytometry**, Zosia Maciorwski
14:00 – 14:45	**Basic data analysis and presentation**, Tomas Kalina
14:45 – 15:15	Coffee break
15:15 – 16:00	**Multicolor panel design**, Zosia Maciorwski
16:00 – 16:45	**FCM diagnostics and functional tests in autoinflammatory diseases – clinical cases**, Alojz Ihan

Saturday, May 5, 2018

	GROUP A	GROUP B	GROUP C	GROUP D
8:30 – 9:15	**Quality Control and Troubleshooting**, Zosia Maciorwski ML			
9:15 – 9:45	Coffee break			
9:45 – 11:45	W5 **Intracellular antibody evaluation** David Hedley, Sue Chow **BC CytoFLEX**	ML **Build your own cytometer** Bill Telford	W3 **Panel development for multicolor flow cytometry**, Tomas Kalina **BD FACSLyric**	W2 **Multicolor flow cytometry – Basic** Zosia Maciorwski **BD CANTO II**
11:45 – 12:45	Lunch			
12:45 – 14:45	W4 **Data analysis and presentation** Claudio Vallan	W2 **Multicolor flow cytometry – Basic** Zosia Maciorwski **BD CANTO II**	W5 **Intracellular antibody evaluation** David Hedley, Sue Chow **BC CytoFLEX**	W3 **Panel development for multicolor flow cytometry** Tomas Kalina **BD FACSLyric**
14:45 – 15:15	Coffee break			
15:15 – 17:15	W1 **Cell sorting basic** Matthias Schiemann **BIO RAD S3**	W5 **Intracellular antibody evaluation** David Hedley, Sue Chow **BC CytoFLEX**	W3 **Stem cell assays** Bill Telford **BD FACSLyric**	W4 **Data analysis and presentation** Claudio Vallan

18:00 Guided tour of Ljubljana

20:00 Social dinner

Sunday, May 6, 2018

	GROUP A	GROUP B	GROUP C	GROUP D
8:00 – 10:00	W5 **Microparticles** Andreas Spittler **BC CytoFLEX**	W3 **Panel development for multicolor flow cytometry** Tomas Kalina **BD FACSLyric**	W4 **Leukemia lymphoma phenotype and minimal residual disease** Hana Glier	ML **Build your own cytometer** Bill Telford
10:00 – 10:30	Coffee break			
10:30 – 12:30	W3 **Panel development for multicolor flow cytometry** Tomas Kalina **BD FACSLyric**	W1 **Cell sorting – Advanced** Matthias Schiemann **BIO RAD S3**	W4 **Data Analysis and presentation** Claudio Vallan	W2 **Cell signalling and phosphoproteins** David Hedley, Sue Chow **BD CANTO II**
12:30 – 13:30	Lunch			
13:30 – 15:30	W2 **Cell signalling and phosphoproteins** David Hedley, Sue Chow **BD FACSCanto II**	W4 **Data analysis and presentation** Claudio Vallan	W5 **Microparticles** Andreas Spittler **BC CytoFLEX**	W1 **Cell sorting – Advanced** Matthias Schiemann **BIO RAD S3**

Basics of Flow Cytometry

Zosia Maciorowski
Curie Institute
Paris, France

What is flow cytometry?

A technology which allows us to measure:

Light scatter

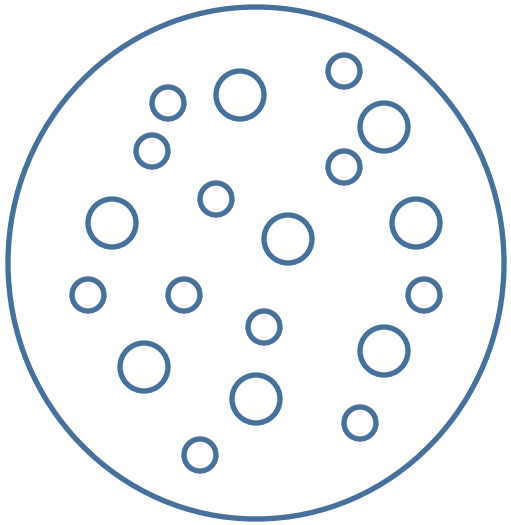
fluorescence intensity

on cells or other particles

one by one (cells are in suspension)

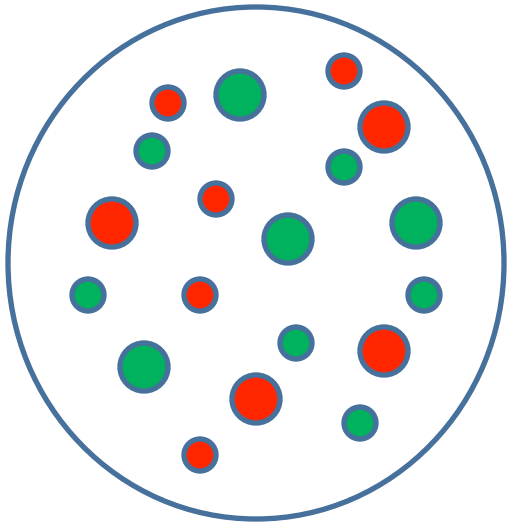
.

When should we use a flow cytometer?



How many Small and/or Big Cells are there ?

Parameter: Size



How many Small cells are Green and/or Red?

How many Big cells are Green and/or Red?

Parameter: Color (Fluorescence)

Courtesy of Dr Krishnamurthy

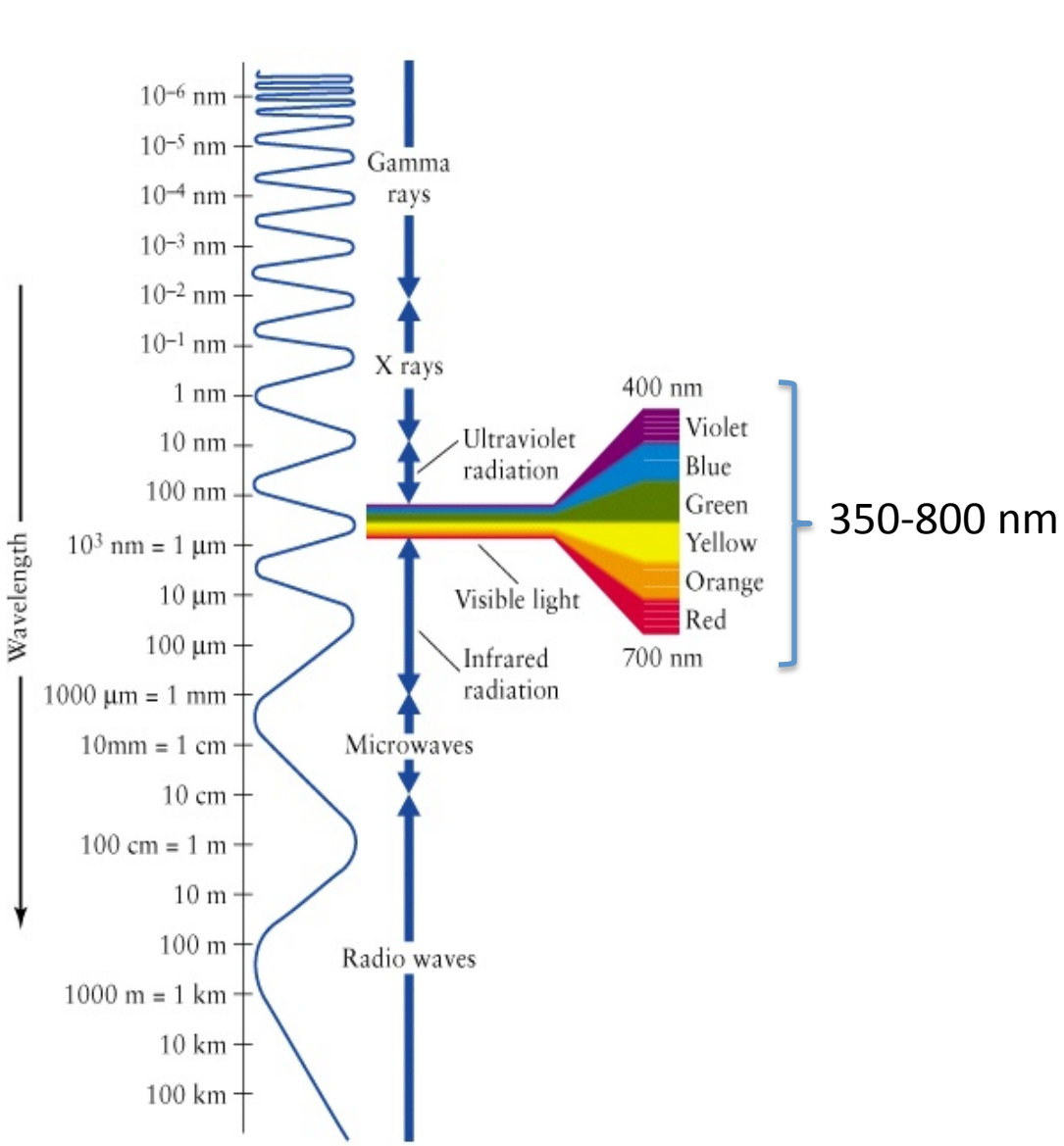
ncbs

TIFR

Overview

- Light
- What we measure:
    - Fluorescence
    - Light scatter
- How a flow cytometer works
    - Fluidics
    - Optics
    - Electronics
    - Cell sorting

Light: the range of wavelengths used in cytometry



350-800 nm

Short wavelength
Higher energy

Long Wavelength
Lower energy

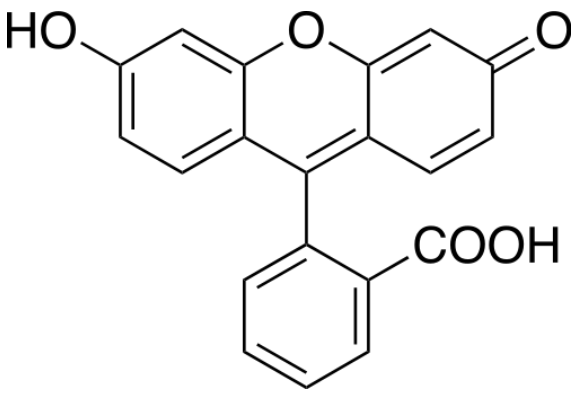
Fluorescence

Fluorochromes

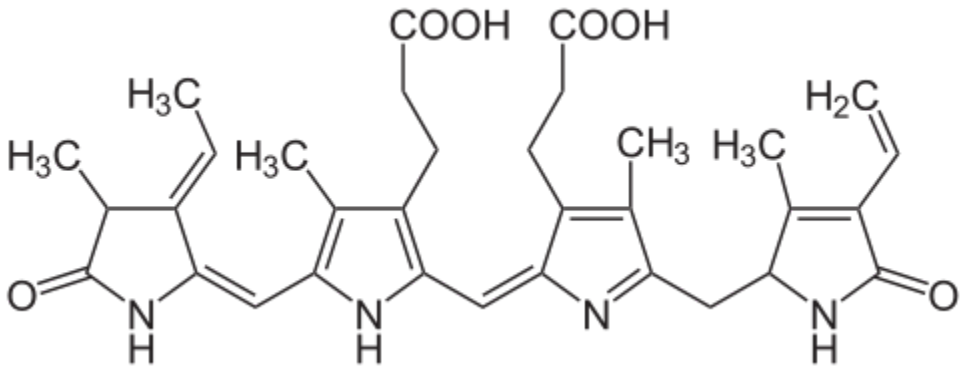
Fluorochromes are molecules
which absorb light at one wavelength
then re-emit the light energy at a longer wavelength

Structures are generally aromatic rings

Fluorescein (FITC)

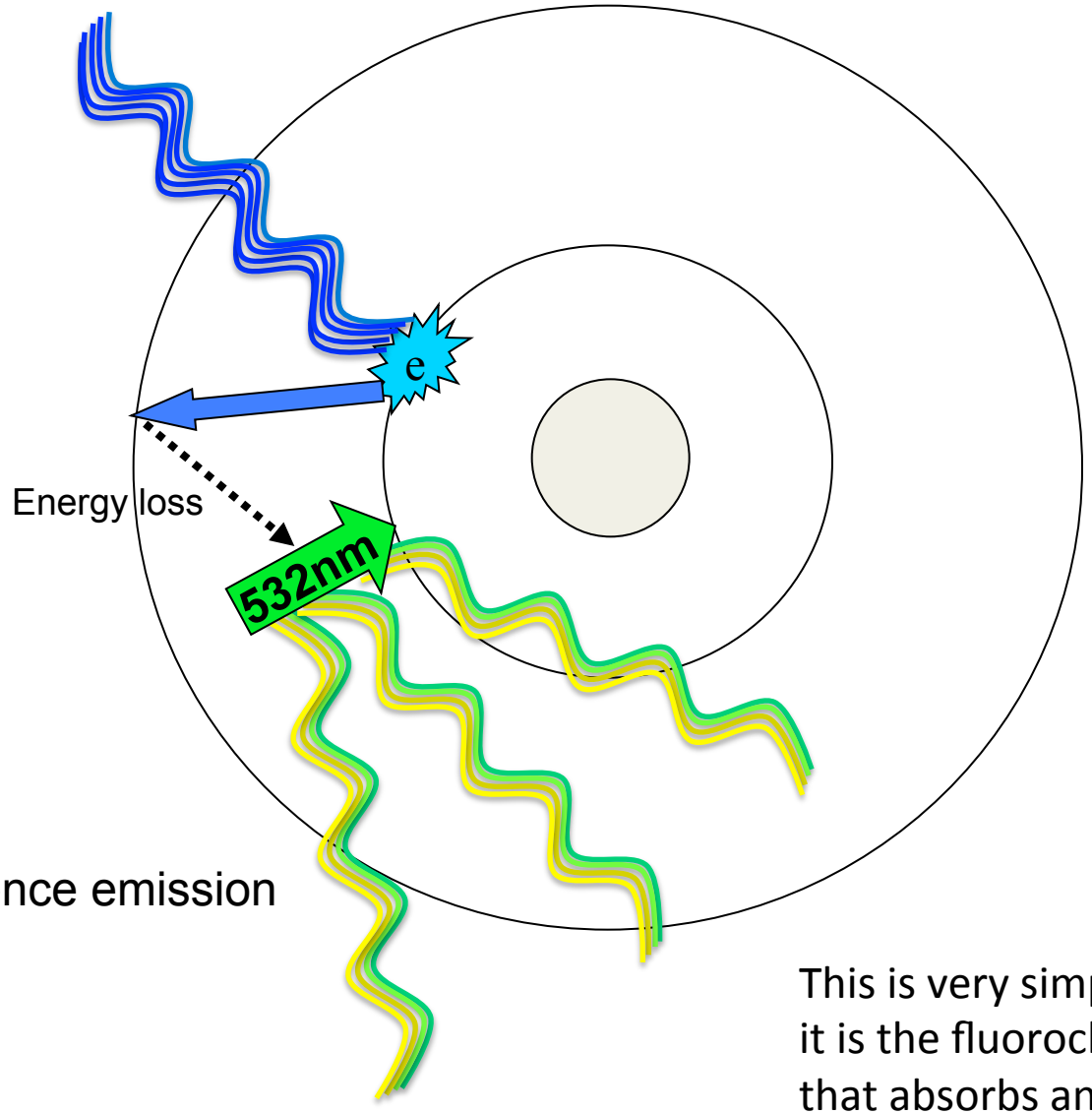


Phycoerytherin (PE)



Fluorescence

Blue 488 Laser excitation

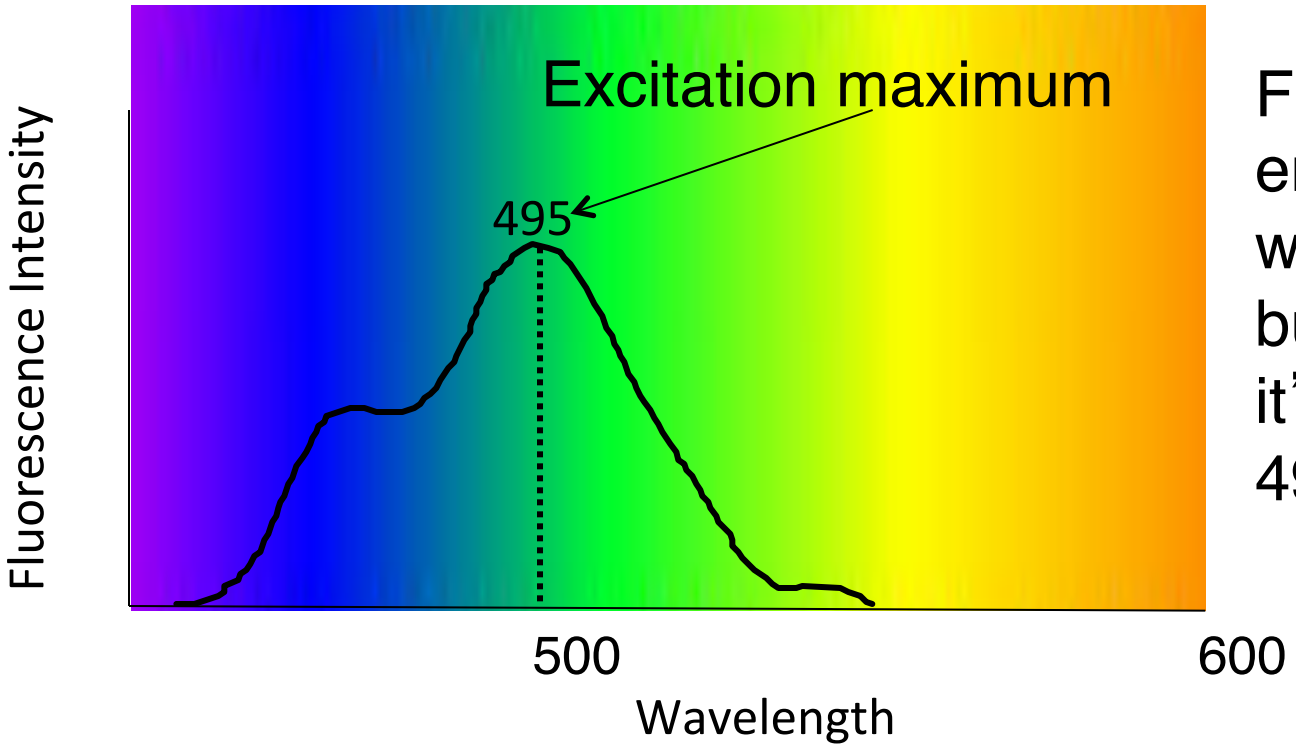


Green fluorescence emission

This is very simplified:
it is the fluorochrome's electron cloud
that absorbs and emits light energy

Excitation spectrum

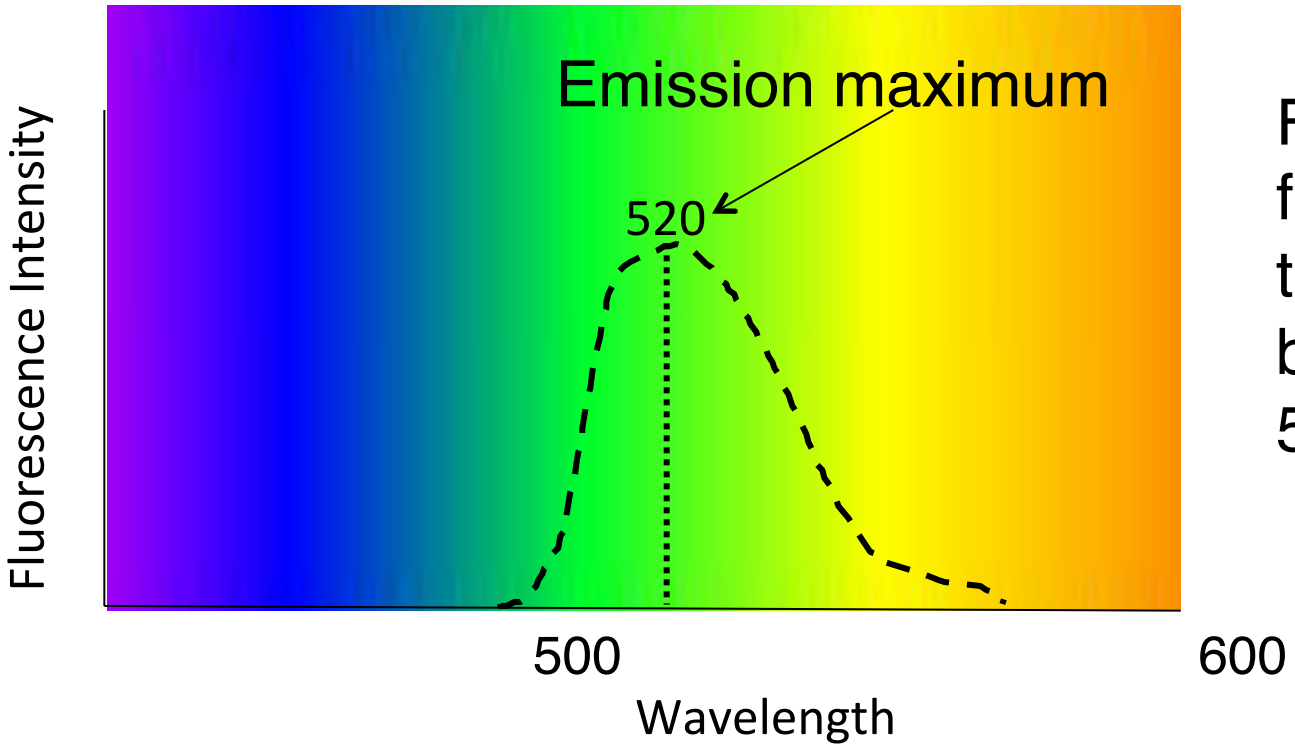
Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths



FITC can absorb energy at all these wavelengths but absorbs best at it's excitation max: 495nm

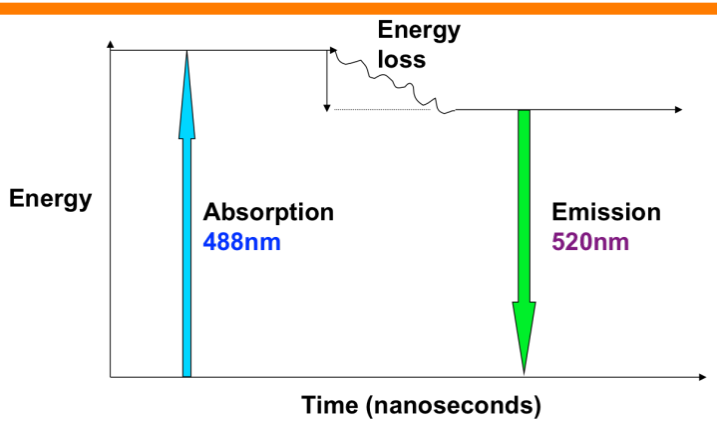
Emission spectra

Each fluorochrome is also capable of emitting light energy over a specific range of wavelengths



FITC will emit fluorescence at all these wavelengths but highest at 520nm

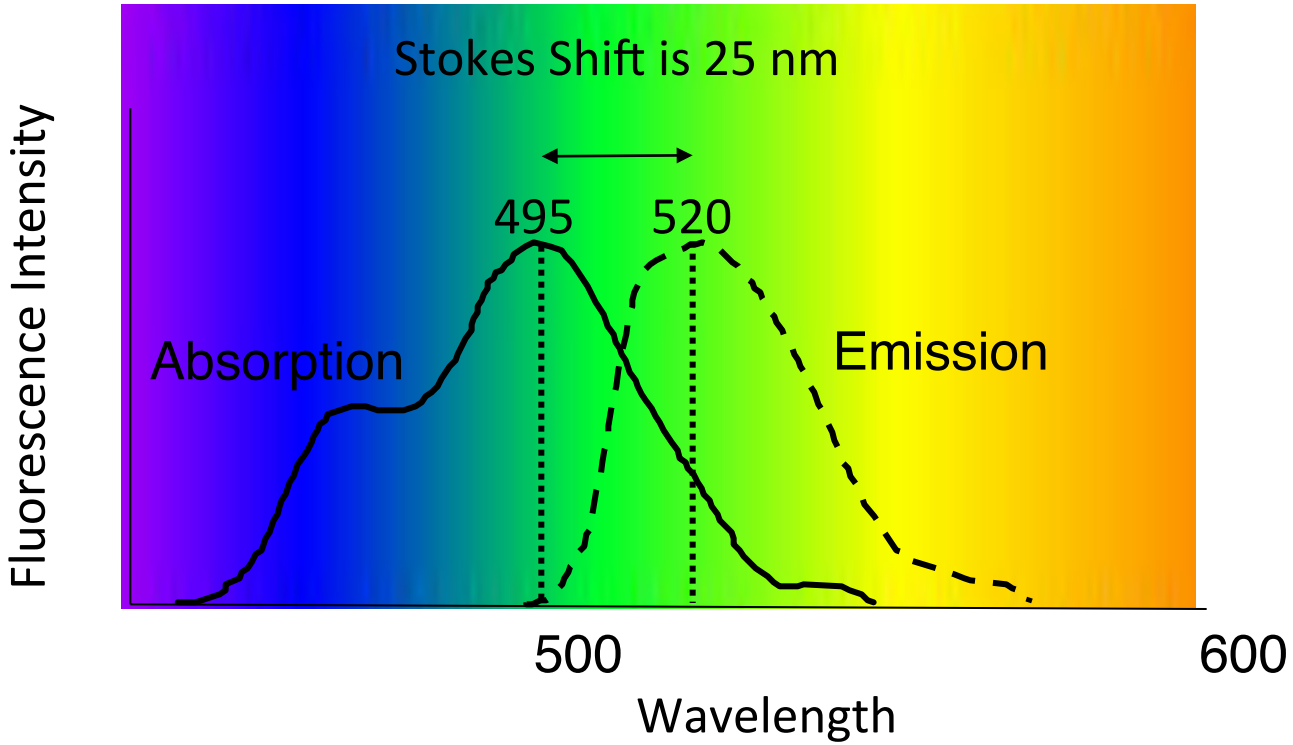
Fluorescence



Stoke's Shift:

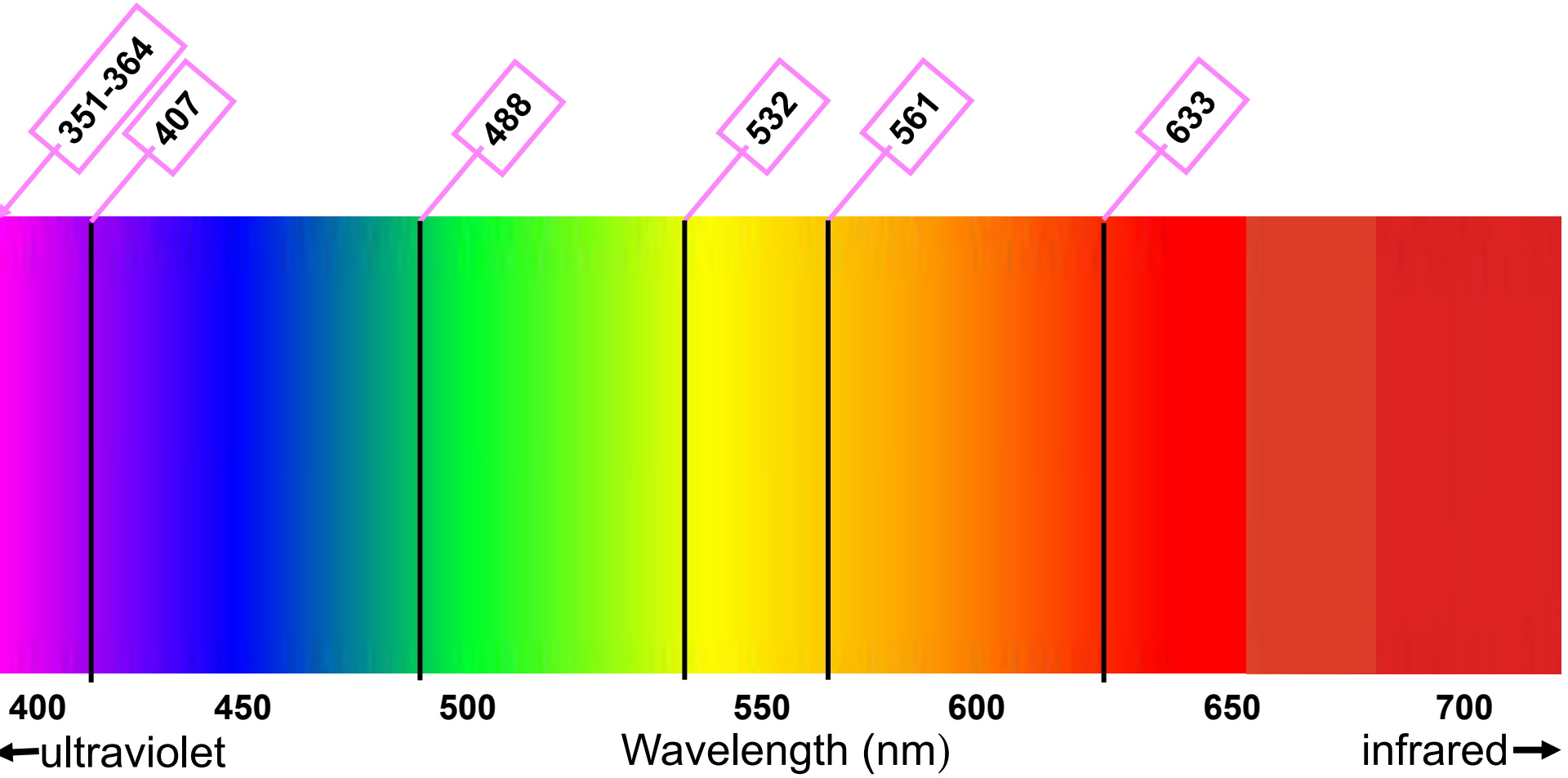
Some of the absorbed energy is lost

Difference between excitation and emission maximum



Laser light is used to excite fluorochromes

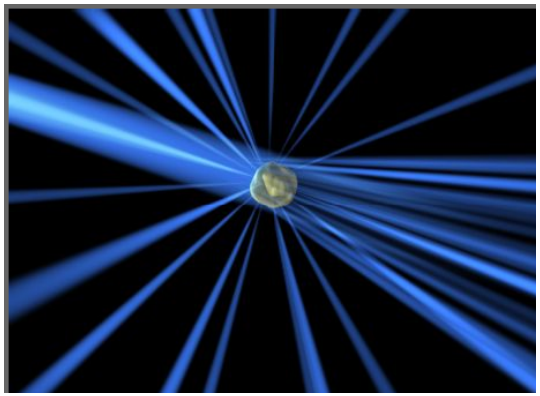
Lasers found on standard flow cytometers

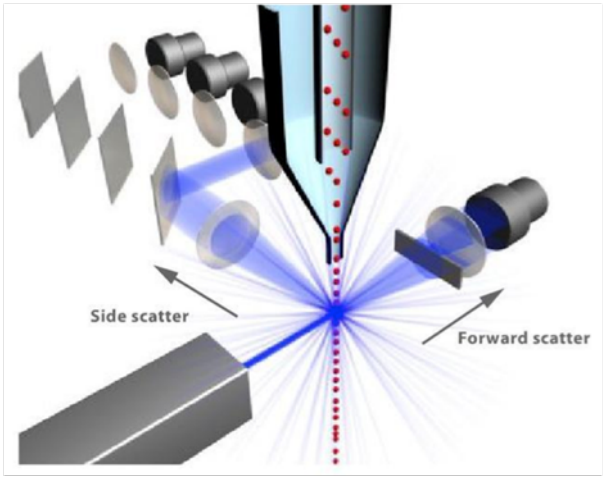


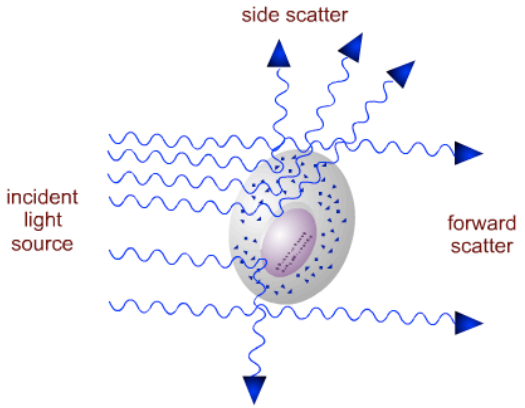
Light Scatter

Light scatter is also measured by flow cytometry

Light scatter is a physical property of the cell or particle which refracts or "scatters" light when it passes a laser beam







Light is scattered in all directions but we measure it at 2 angles:

Forward scatter (FSC): light scattered in the axis of the laser beam

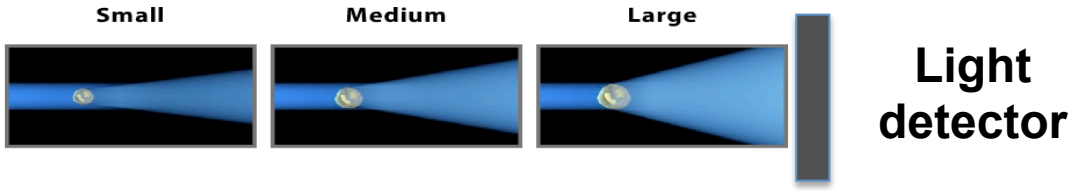
Side scatter (SSC): light scattered at a 90° angle to the laser beam.

Images from Life Technologies Flow Cytometry Tutorials

Courtesy of Kylie Price
Malaghan Institute

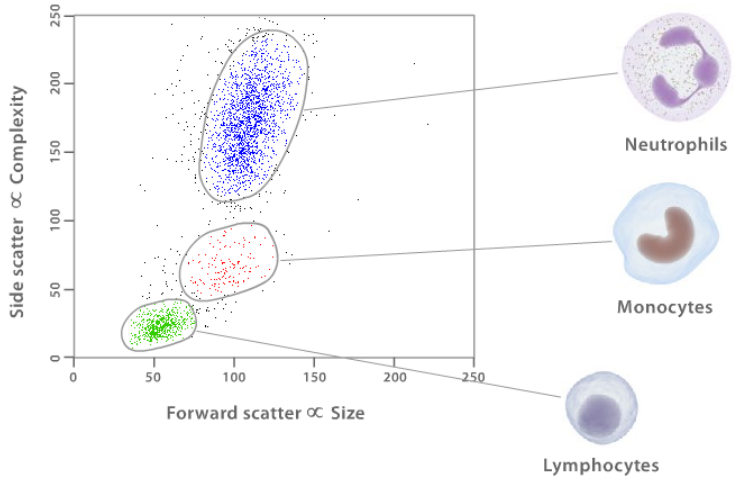
What does light scatter tell us?

Forward scatter is roughly proportional to cell surface properties and size



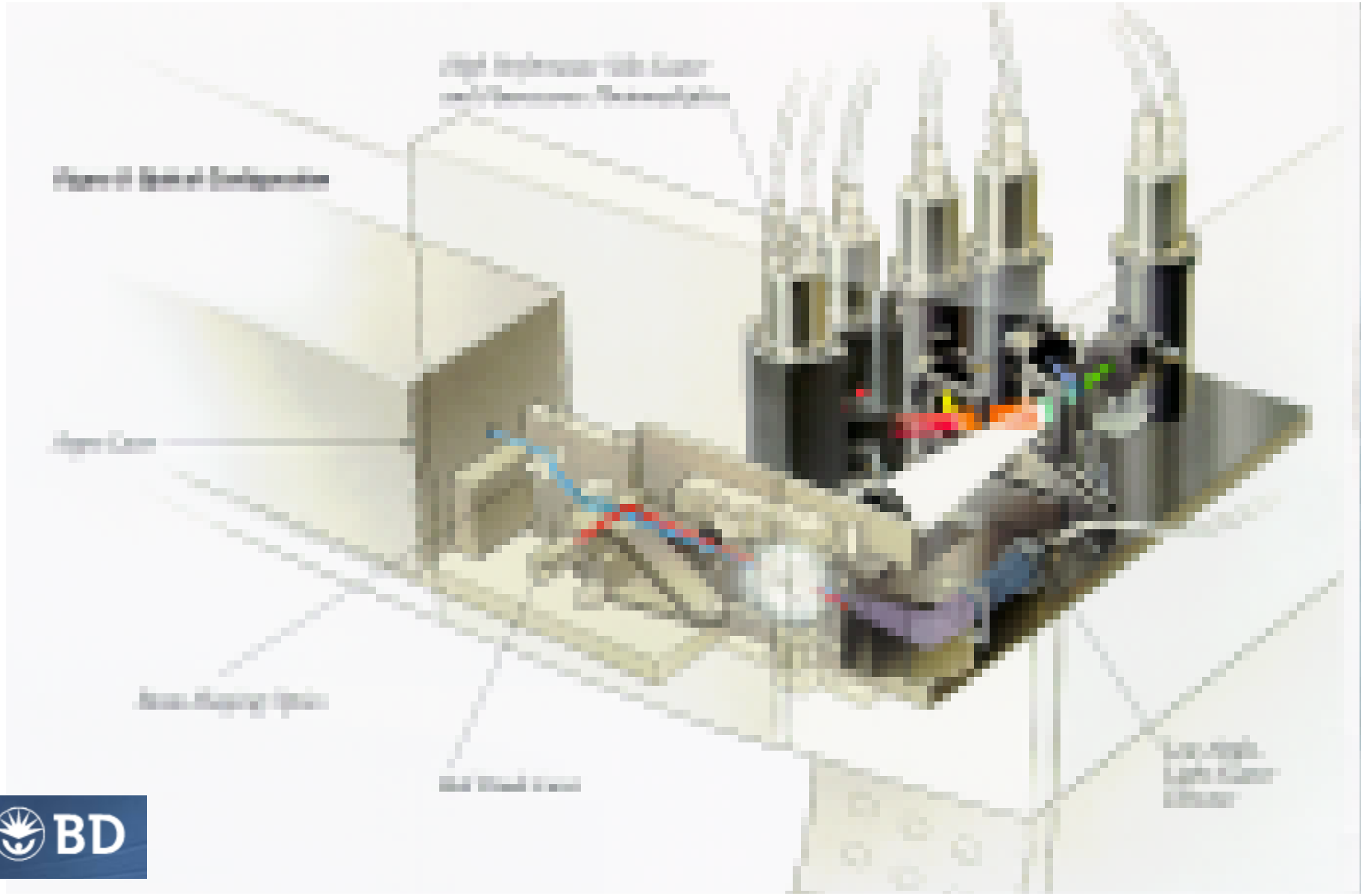
Side Scatter is affected by cell structural complexity and granularity

Neither of these are can be used to quantitate the size of cells, however they can be used to distinguish different types of cells



Courtesy of Kylie Price
Malaghan Institute

It’s not a black box!

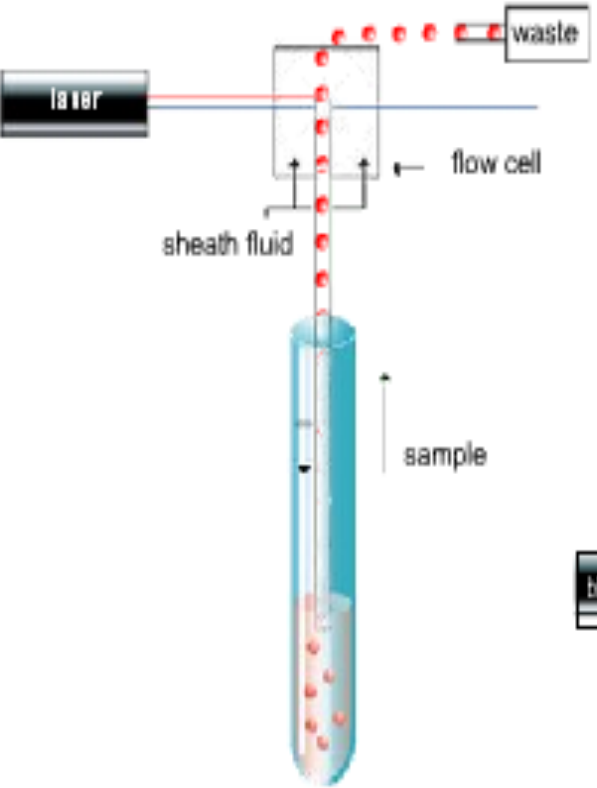


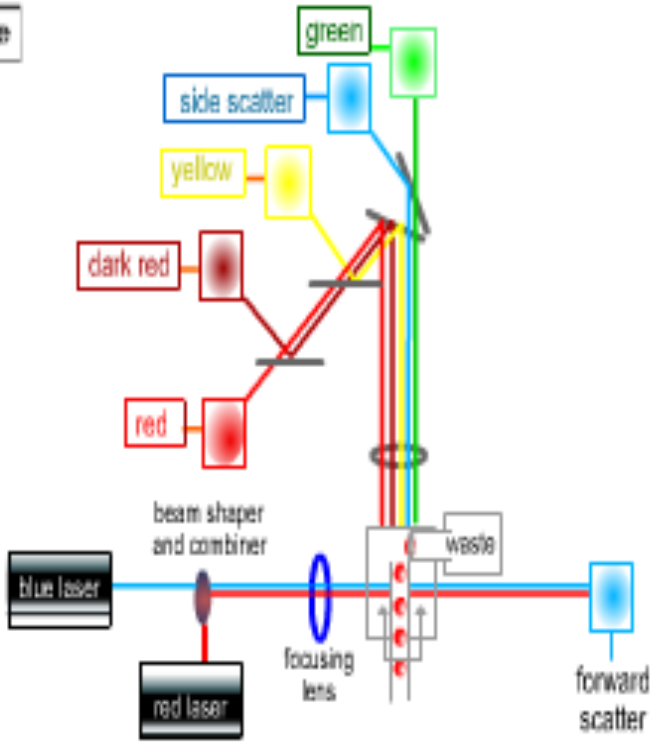
What do you find inside a Flow Cytometer?

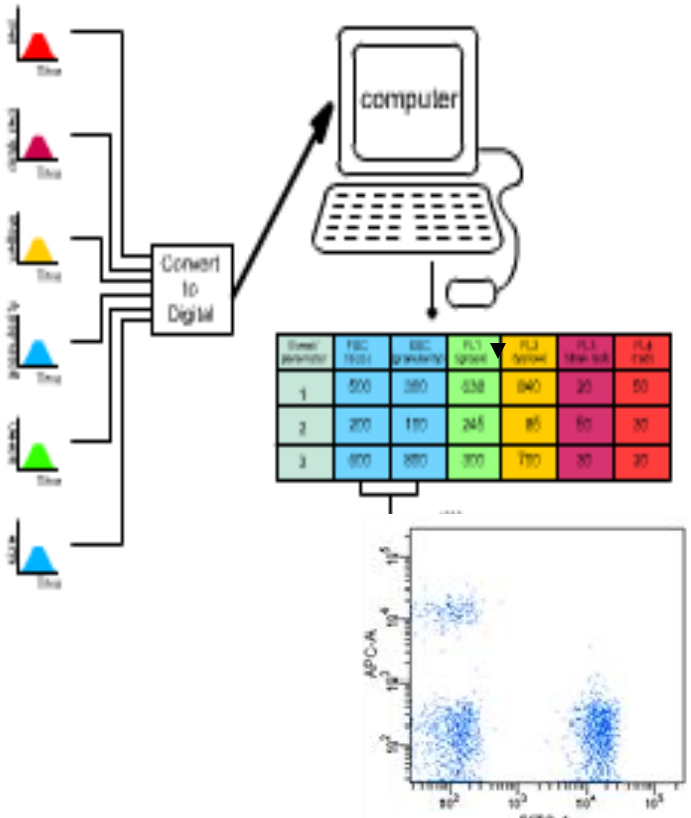
Fluidics
Position cells
to flow one by one
past the laser beam

Optics
Separate the light emission
from different fluorochromes
and direct towards
detectors

Electronics
Detectors convert light
emission to voltage pulses
which are digitalized







What do you find inside a Flow Cytometer?

Fluidics
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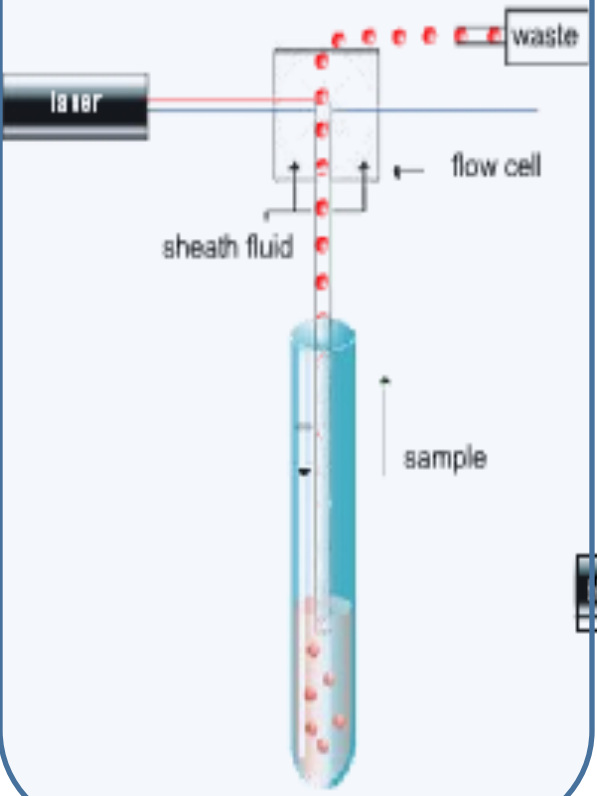
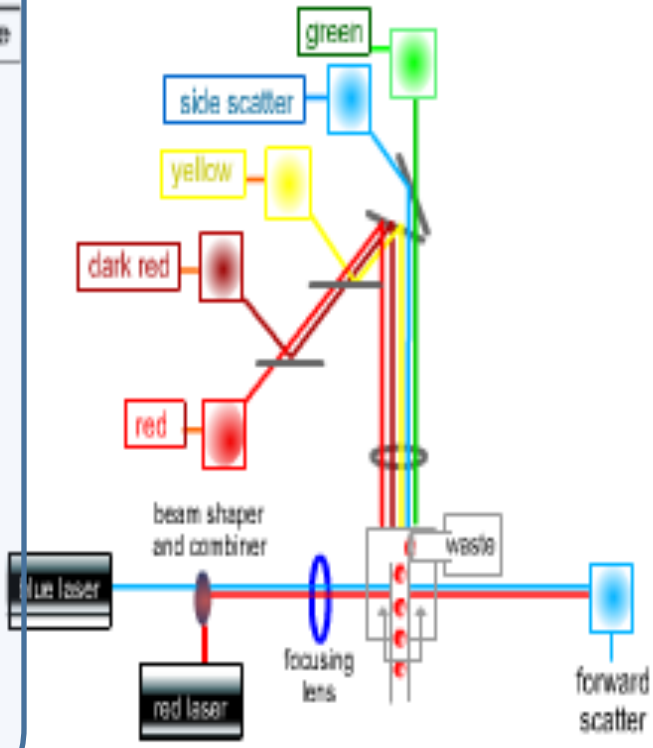
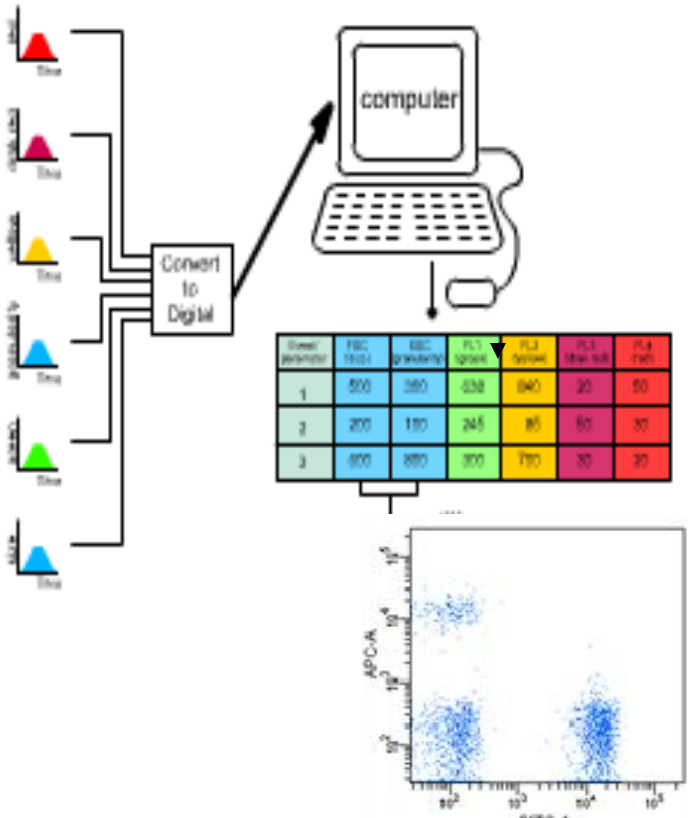


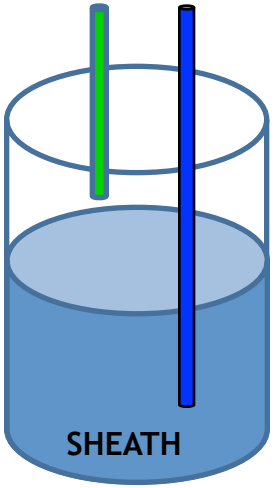
Optics
Separate the light emission from different fluorochromes and direct towards detectors

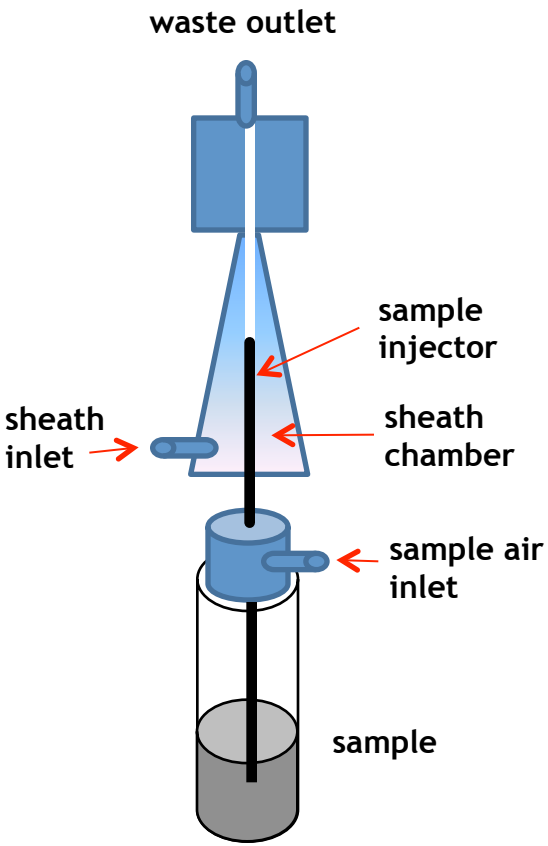


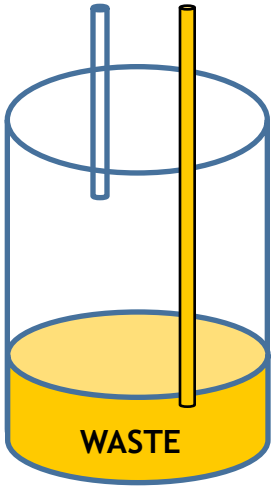
Electronics
Detectors convert light emission to voltage pulses which are digitalized



Instrument Fluidics: positive air pressure system

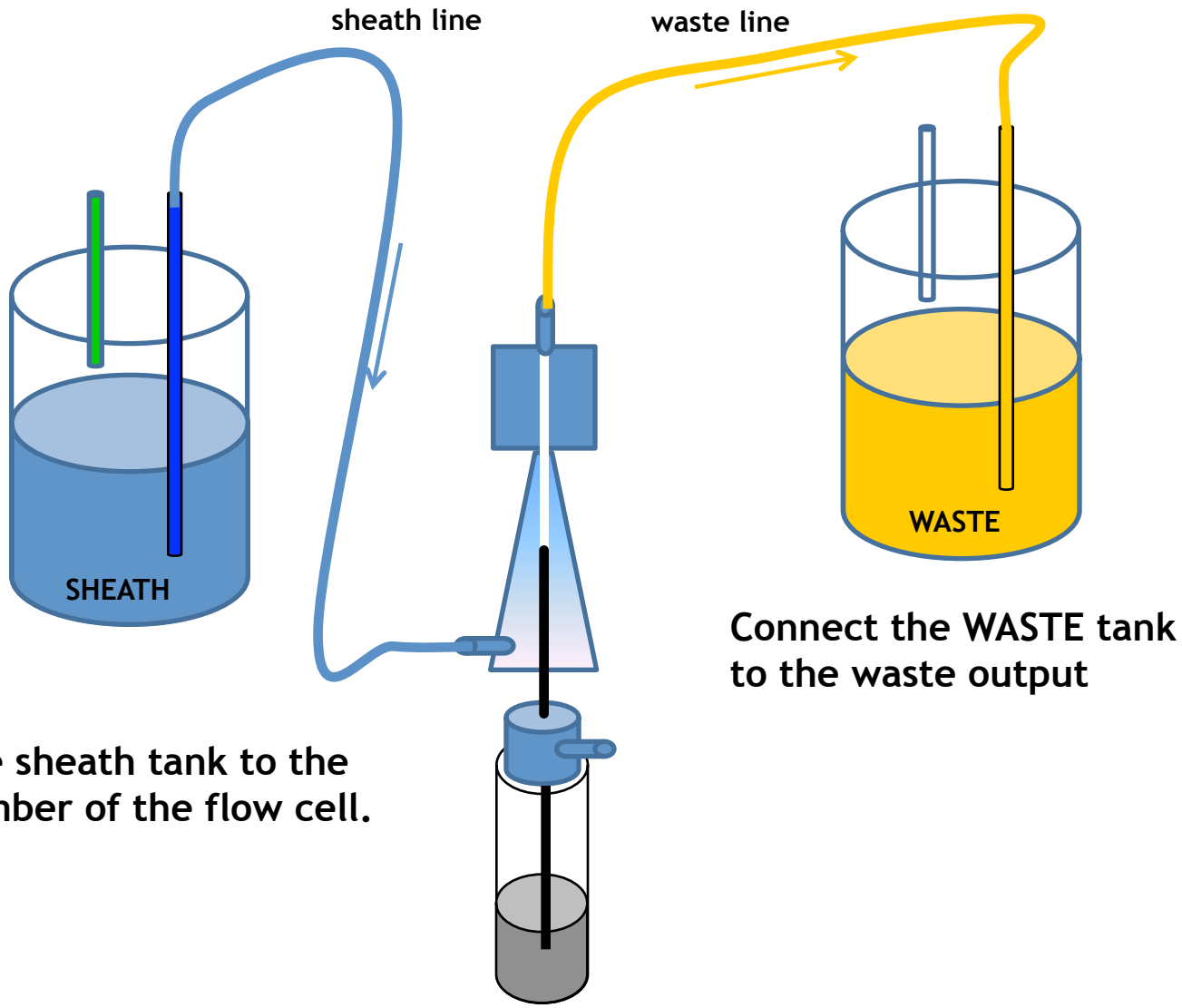






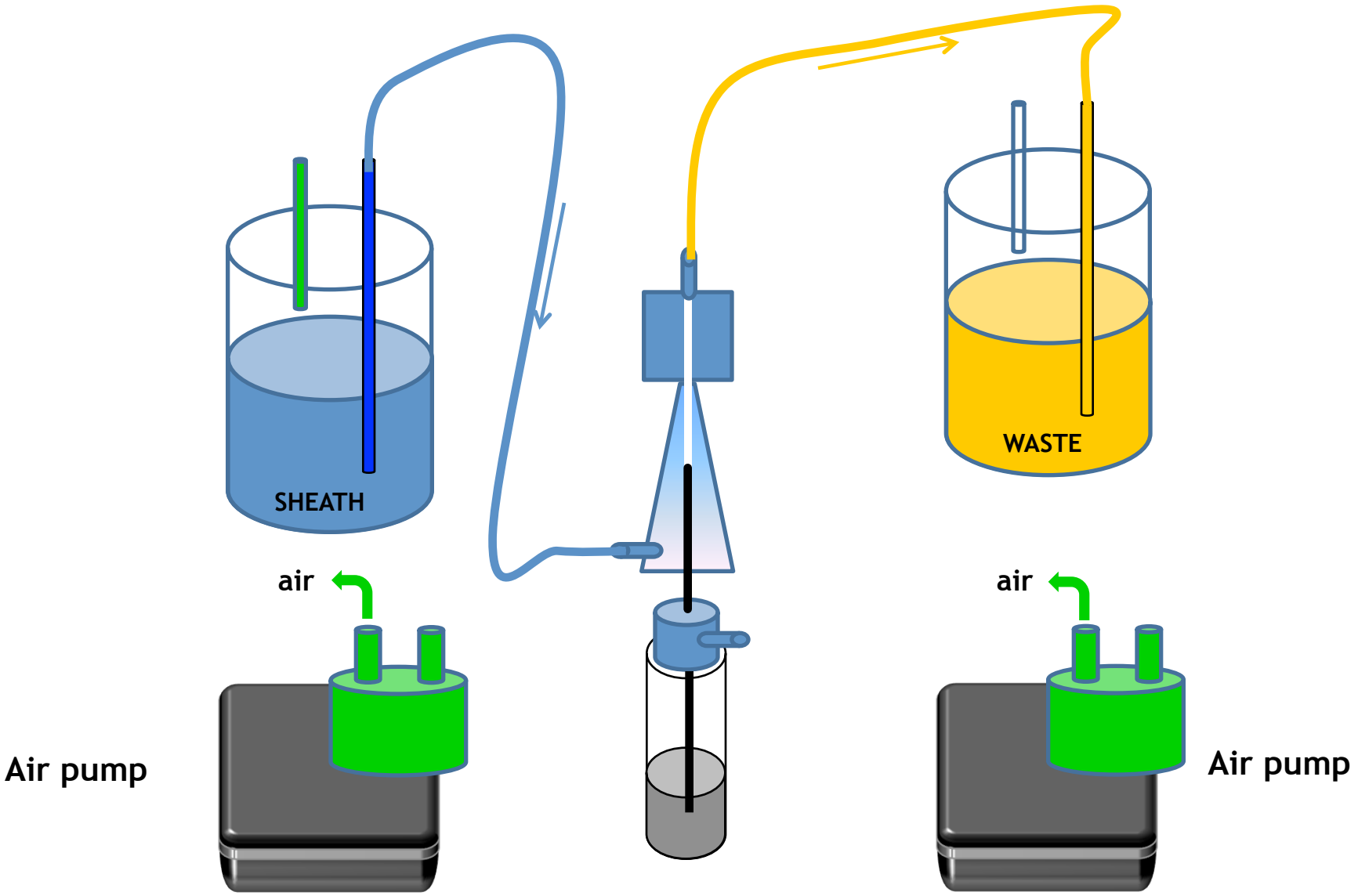
Sheath fluid can be
a saline solution, PBS, water

Slides courtesy of Bill Telford NIH

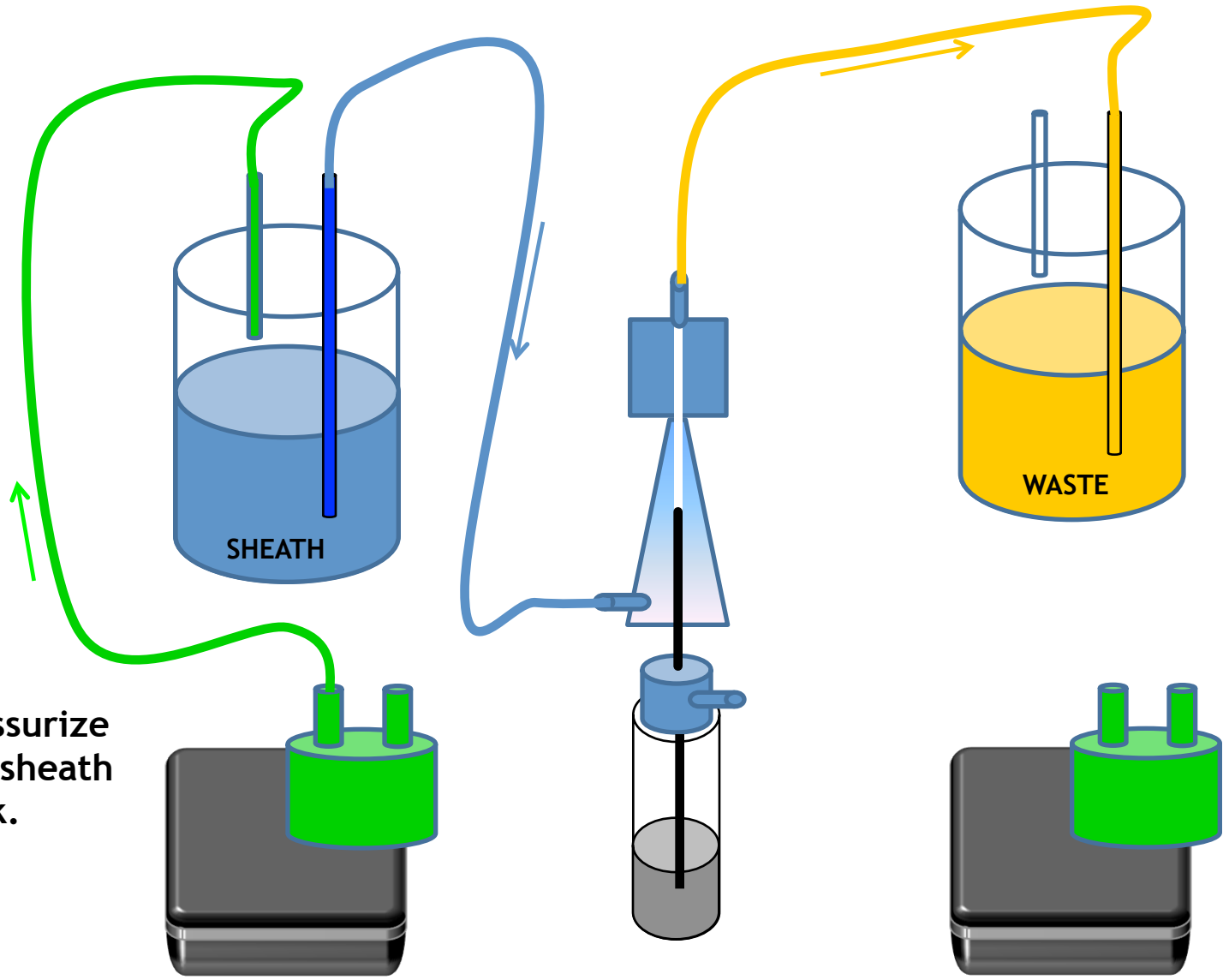


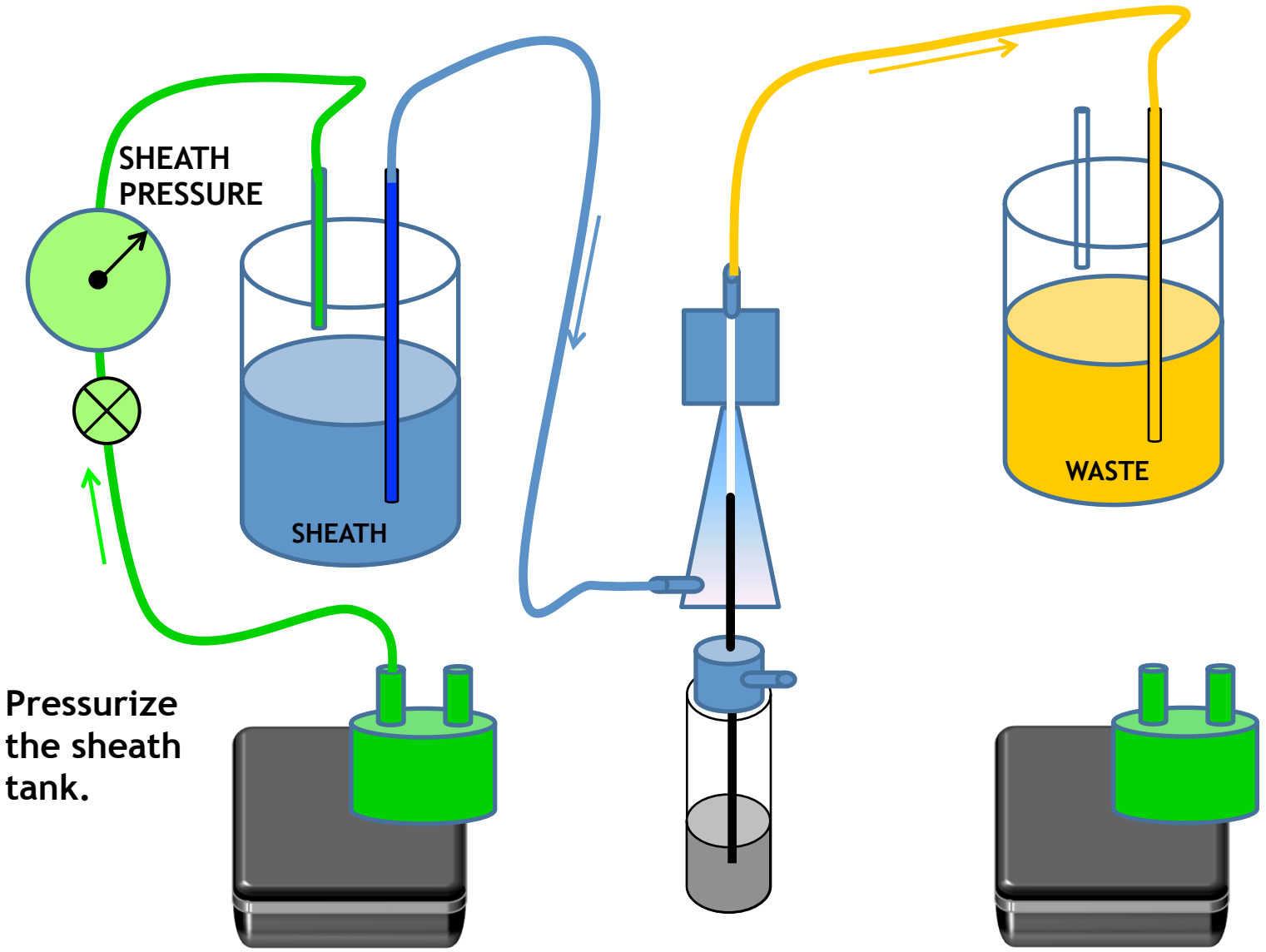
Connect the WASTE tank to the waste output

Connect the sheath tank to the sheath chamber of the flow cell.

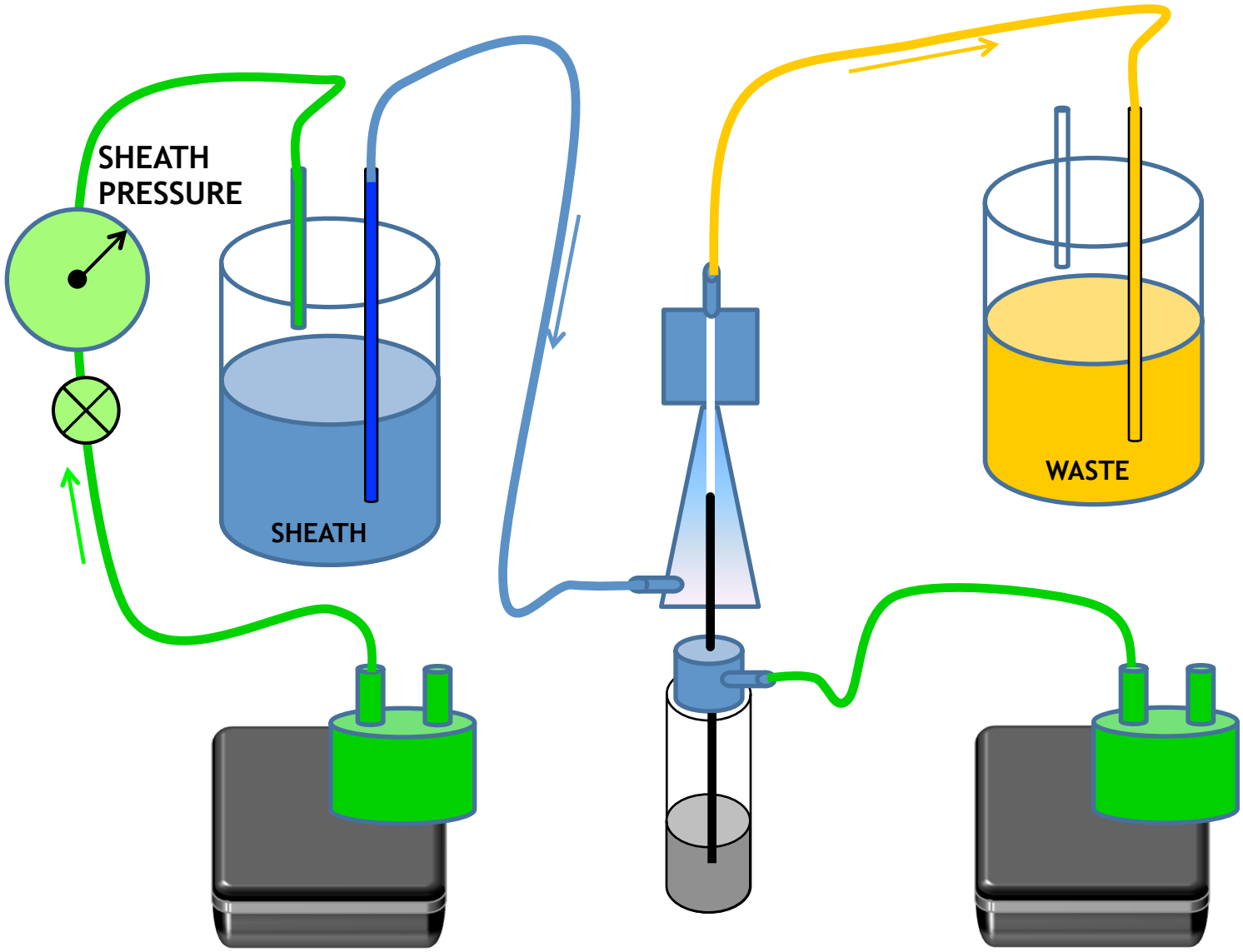

SHEATH
WASTE
air
air
Air pump
Air pump

Pressurize the sheath tank.

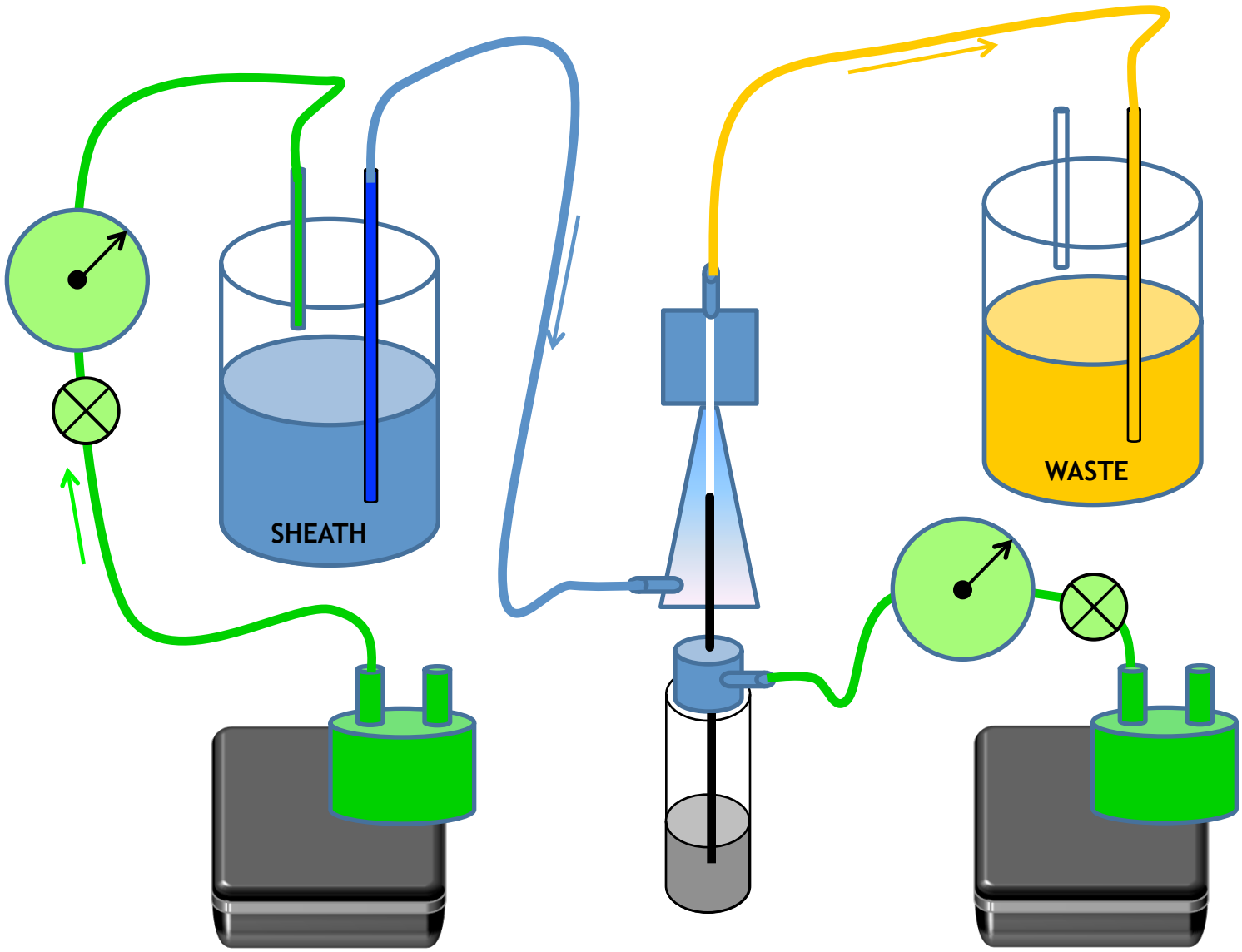




Pressurize the sheath tank.



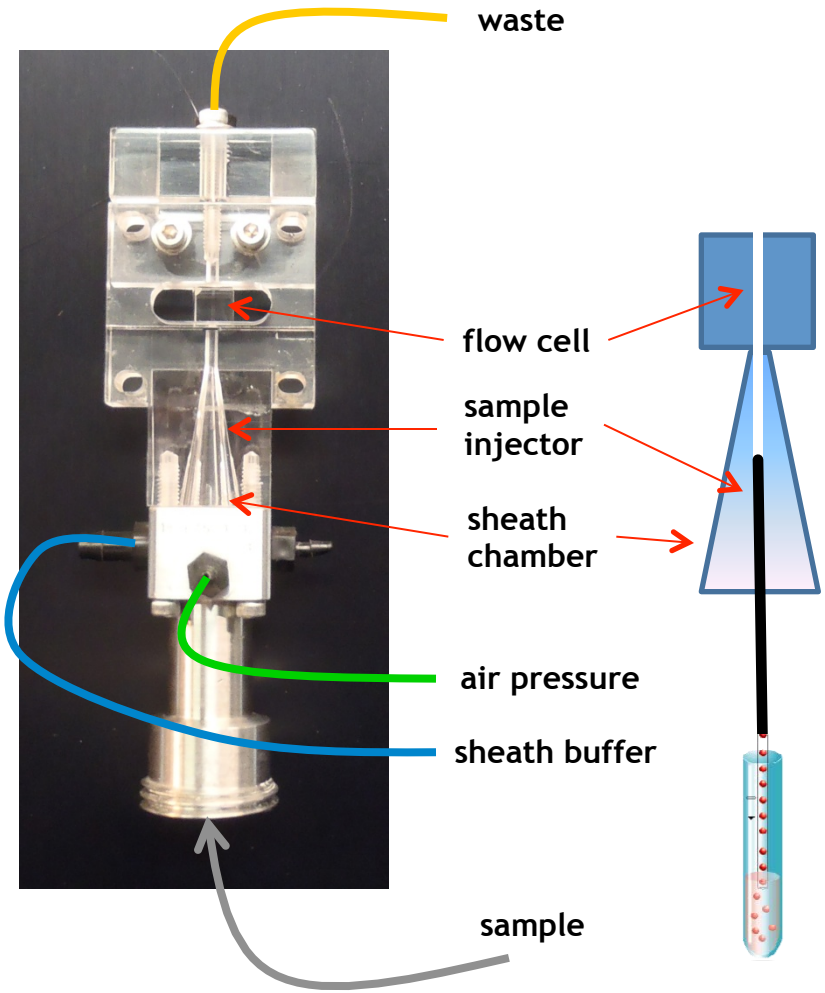
Pressurize the sample port.

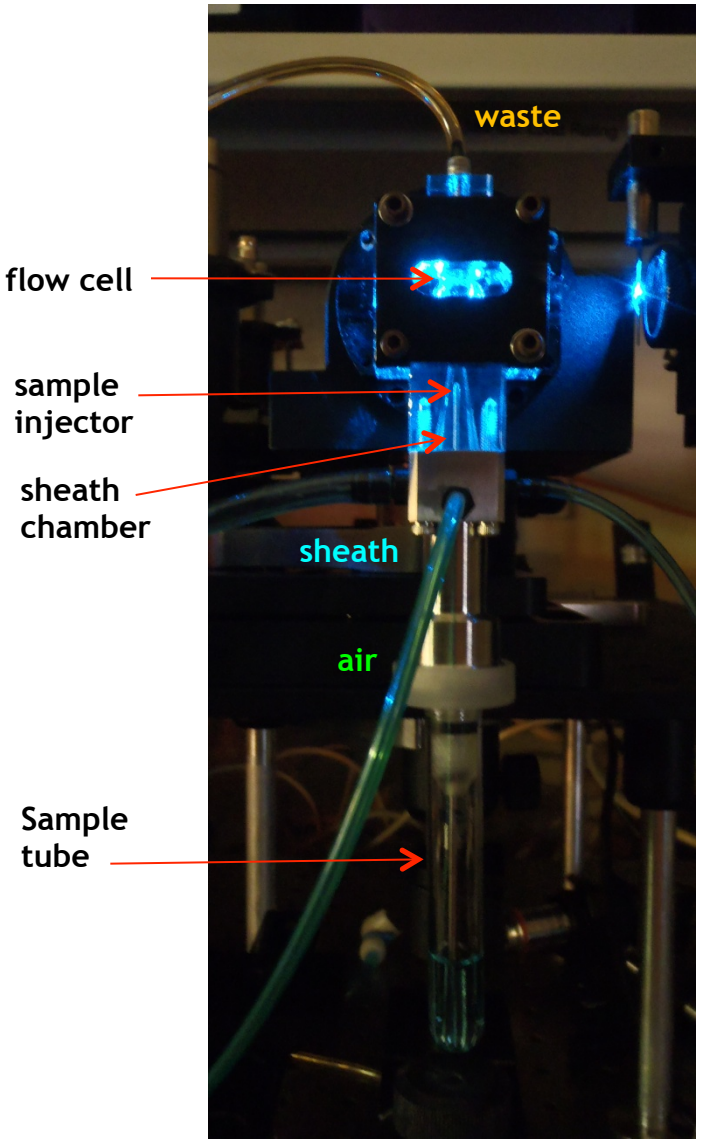


This is very simplified! Most commercial systems have complex pressure regulation mechanisms to carefully control sheath and sample delivery.

What a flow cell looks like

BD LSRII, Fortessa, FACSCalibur

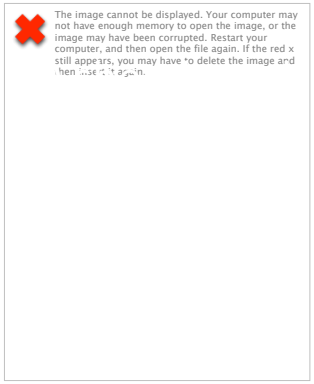




Slide from Bill Telford NIH

Different ways to pump sheath and sample through the cytometer

1. Positive air pressure (which we've just seen)
   - LSRII, Fortessa, Calibur
   - Gallios
   - Sorters (Aria, Astrios, S3, etc)



2. Syringe pump
   - Guava
   - Attune
   - Novocyte (sample)

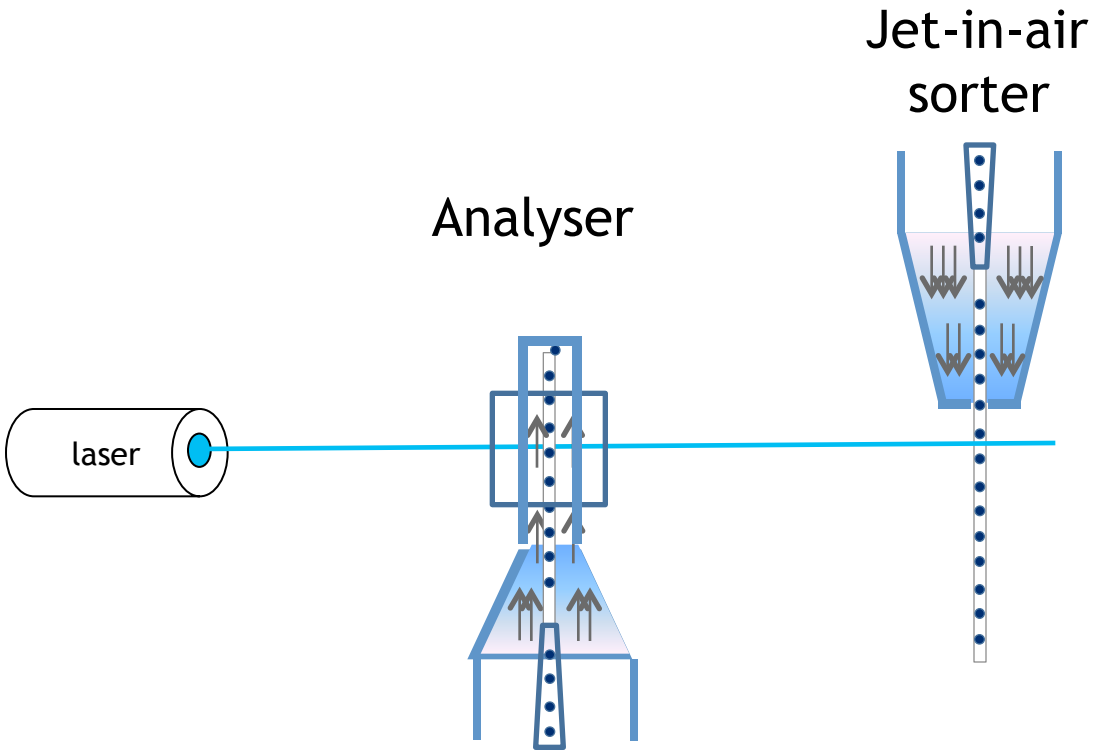
3. Peristaltic pump
   - Accuri
   - Cytoflex
   - Novocyte (sheath)
   - ZE5

Intercepting the sample stream with a laser

The laser beam is focused on the point in the sample stream where the cells will be analyzed.

On an analyser, this is inside the flow cell

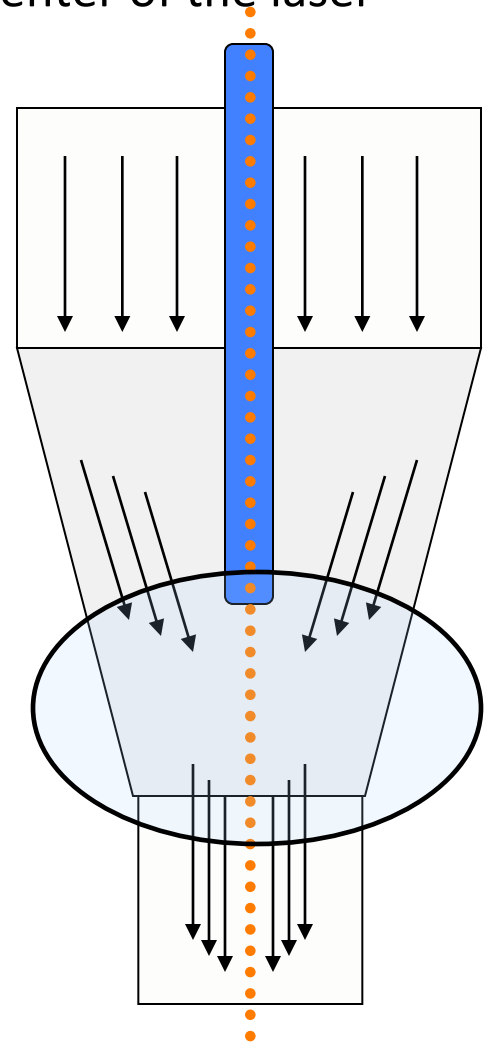
On a jet-in-air sorter, this is just below the nozzle

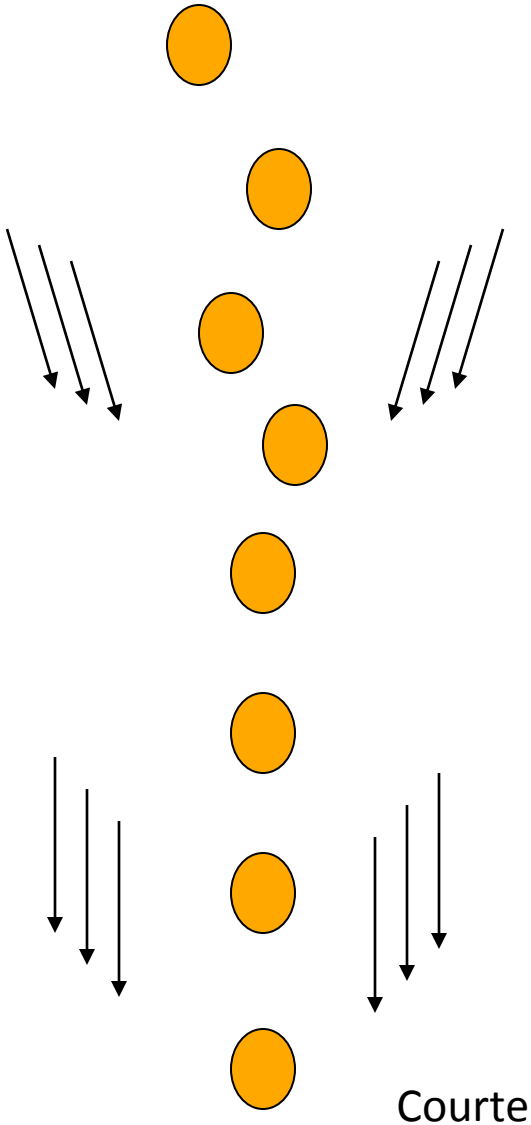


Slide courtesy of Bill Telford

Stream within a Stream: the role of hydrodynamic focusing

Cells are injected into the center of the sheath fluid so that they will be positioned in the center of the laser



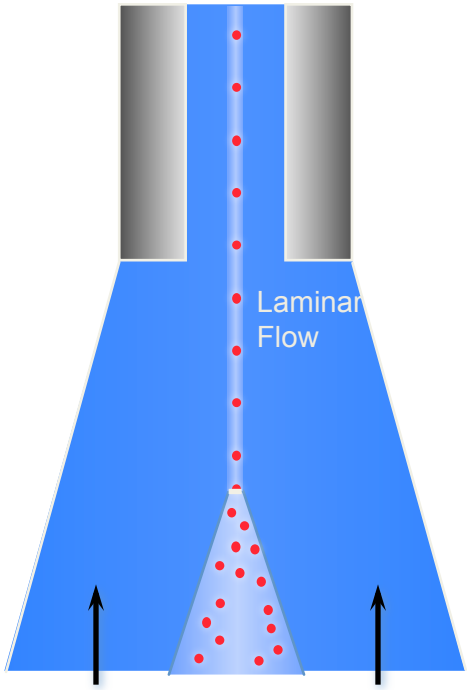


Courtesy of Alan Saluk

The effect of changing the sample pressure

Cytometer **sheath pressure** always remains **fixed**!

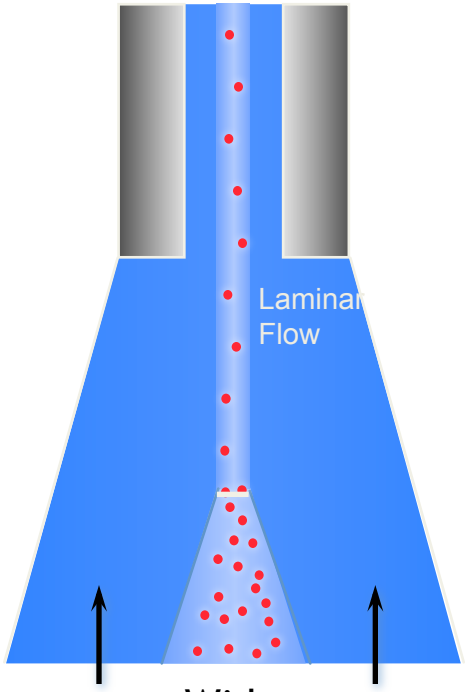
Low sample pressure



Narrow core

All cells pass through center of laser beam
Excitation and emission very uniform
Important to use low for DNA cell cycle analysis!

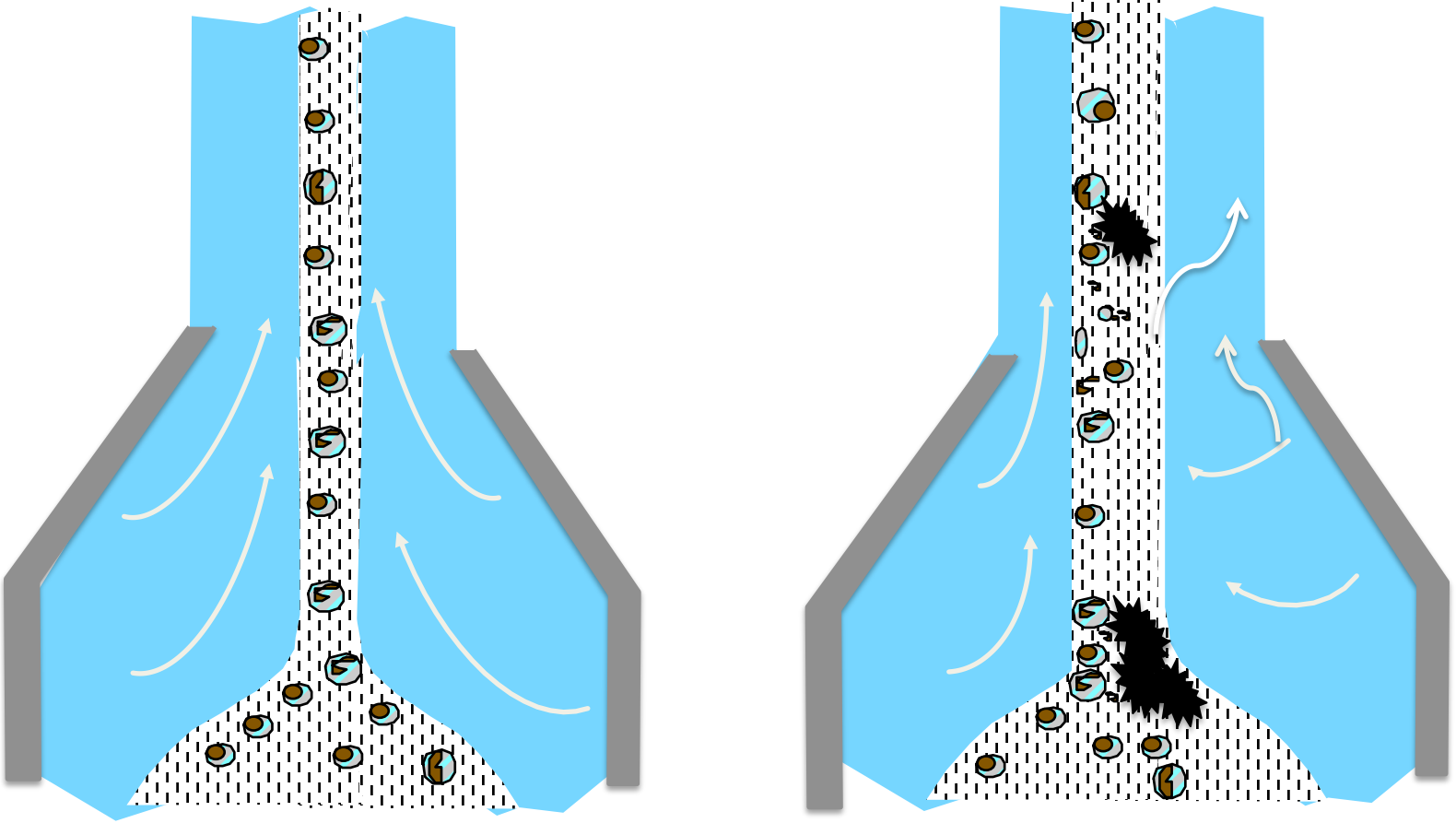
High sample pressure



Wide core

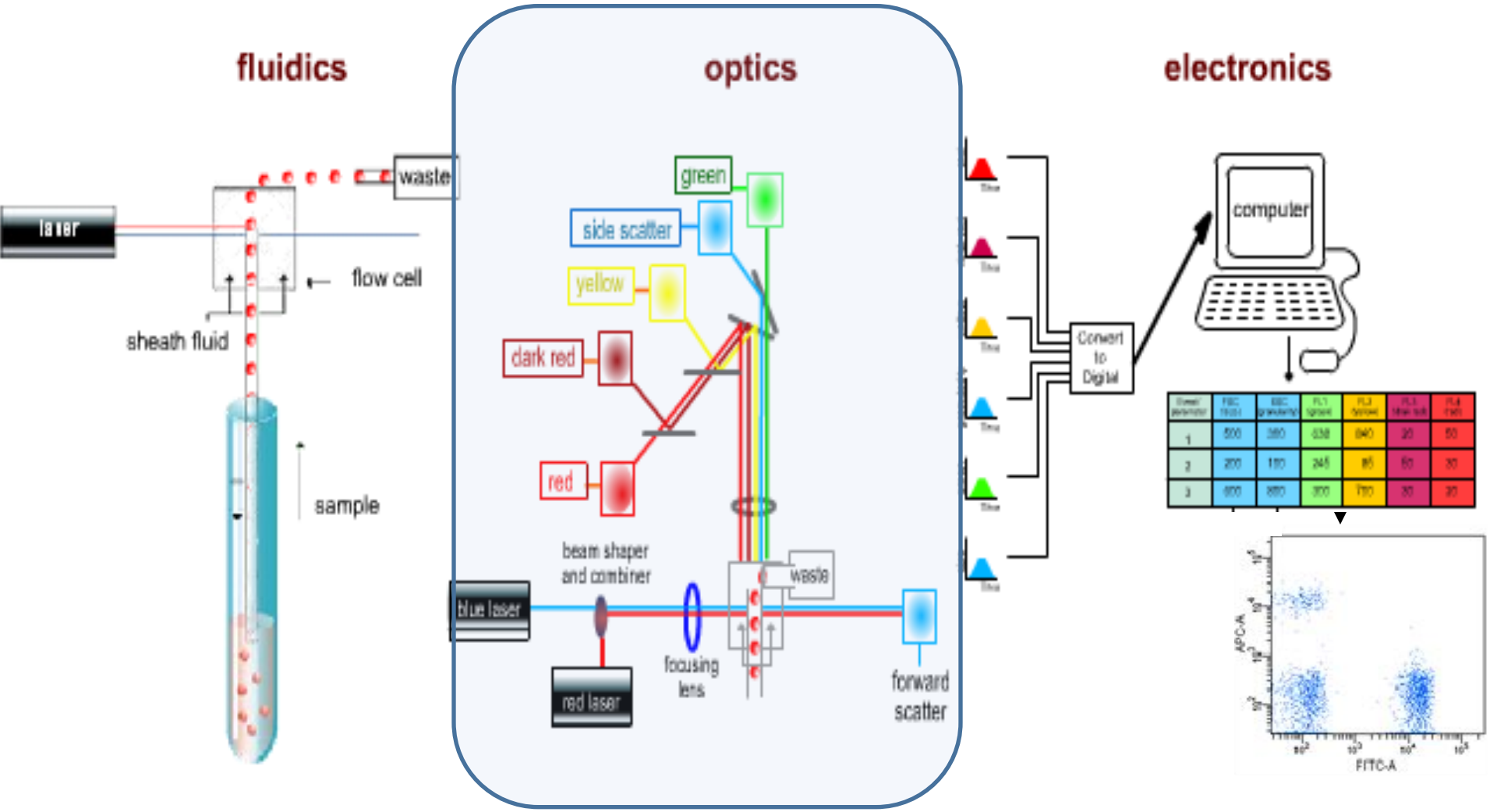
Not all cells pass through center of laser beam
Excitation and emission not uniform

Air bubbles or dirt will decrease signal



Courtesy of Grace Chojnowski

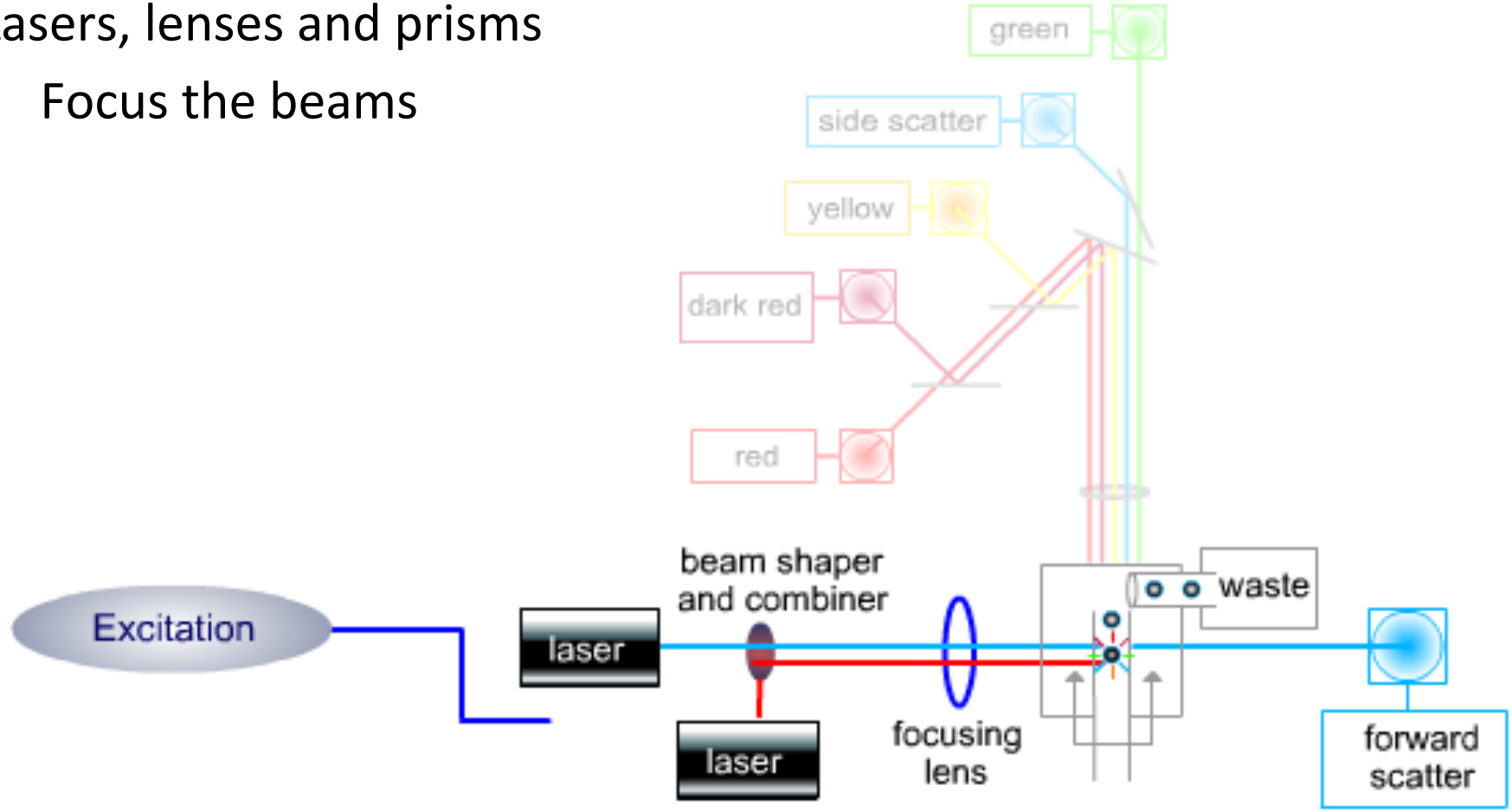
Flow Cytometer Elements



Excitation Optics

Lasers, lenses and prisms

Focus the beams



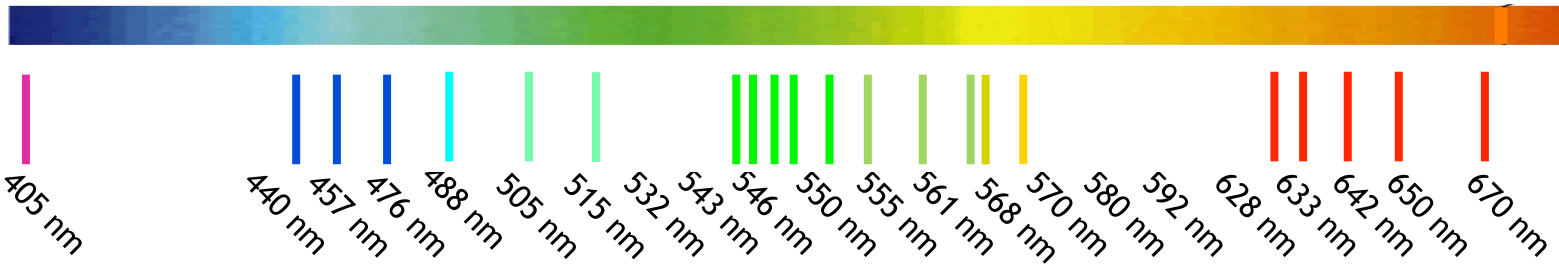
Let there be Light!

Laser characteristics

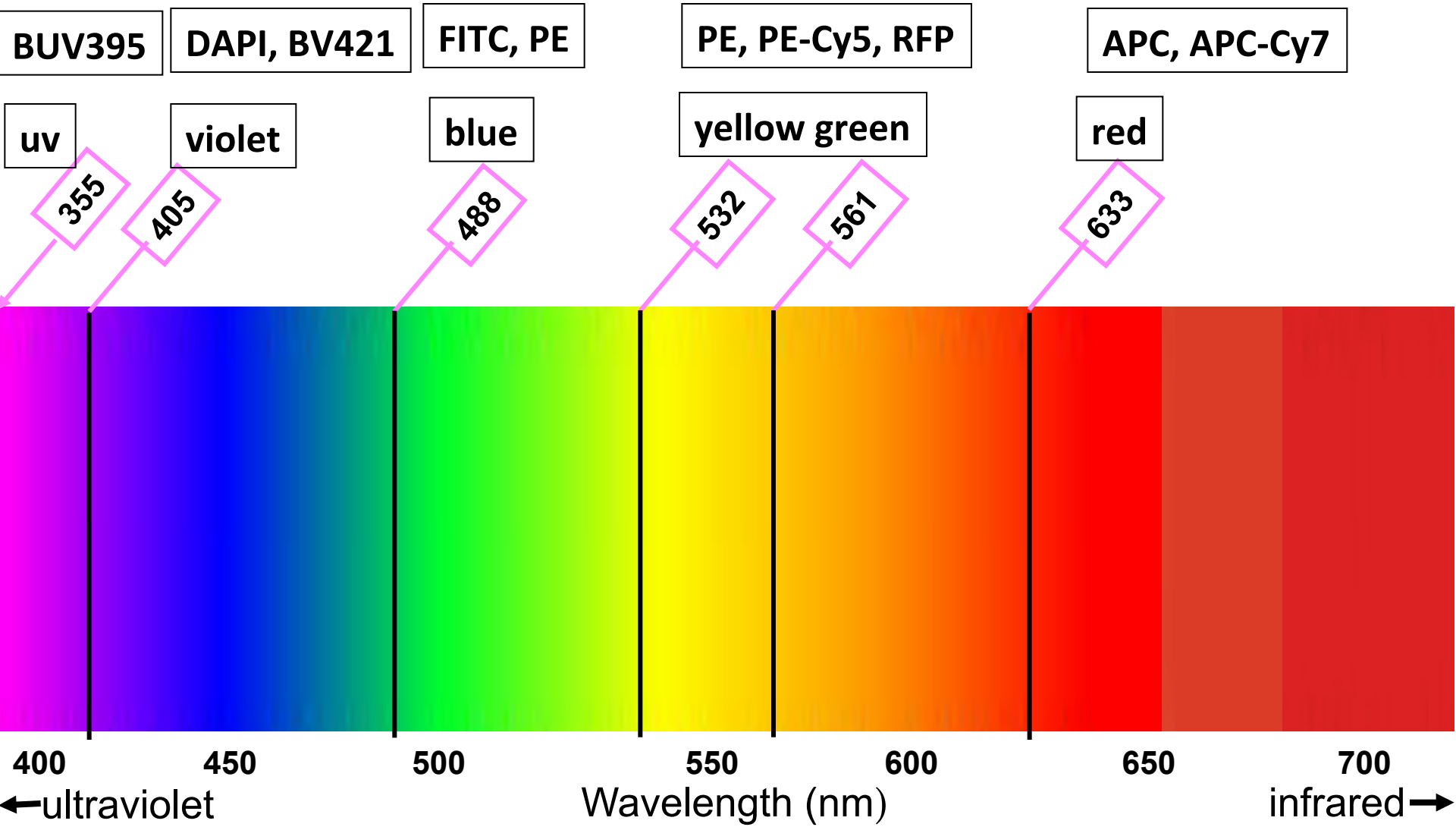
Bright
Coherent
Emit at a single wavelength
Stable
Focus to a tight spot on a tiny area
(like a sample stream)
getting smaller and cheaper!

New Generation Solid State Lasers

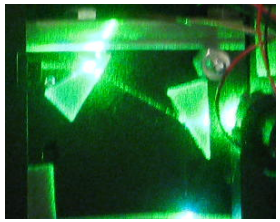
available in virtually **any color** allowing excitation of almost any fluorescent molecule

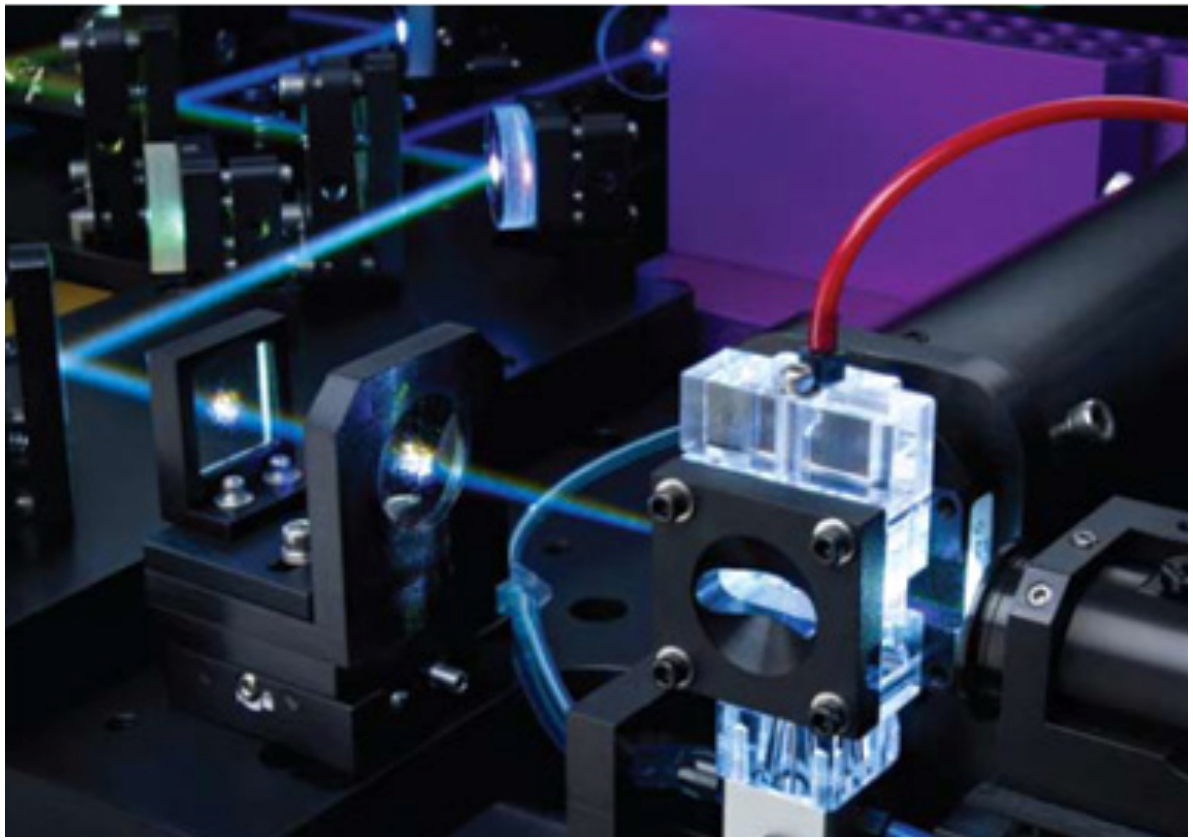


Laser wavelengths on typical cytometers



Lenses and prisms direct and focus the laser beams on the cells as they pass through the flow cell





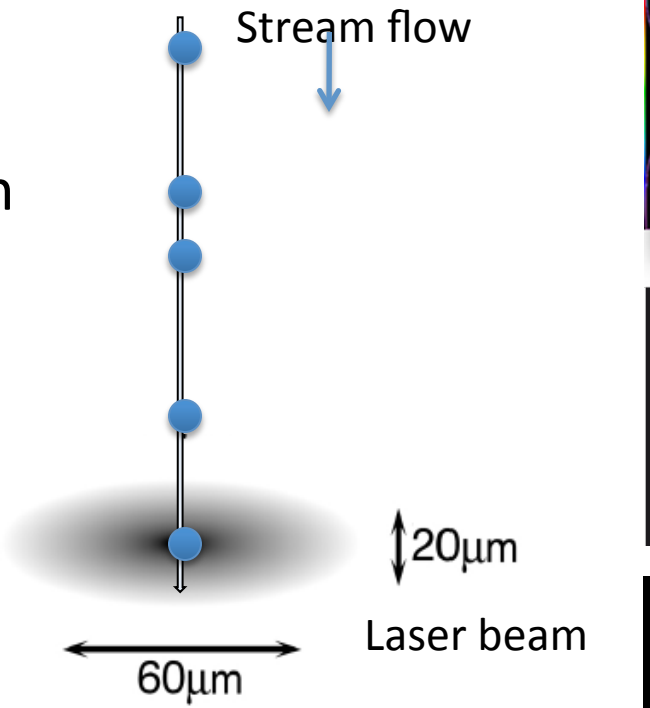
Here we can see a blue laser beam, a violet, a green and a red

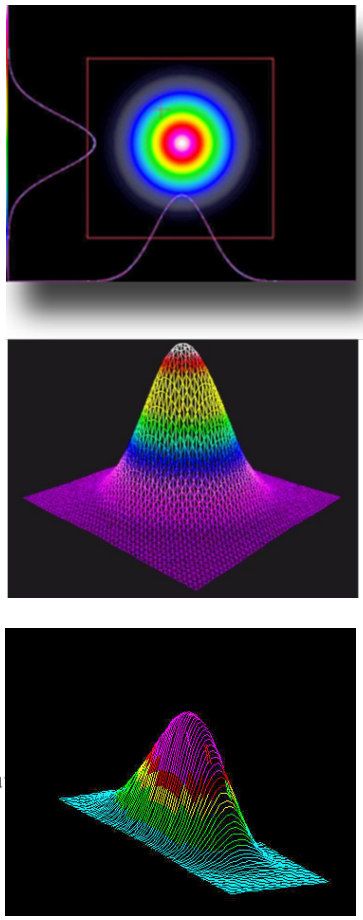
Laser beam geometry

Cells MUST pass through
- center of the laser beam
- for maximum uniform excitation

If they don't:
Decreased excitation means
Decreased fluorescence

Dirt or bubbles can cause this by deflection of the cell path



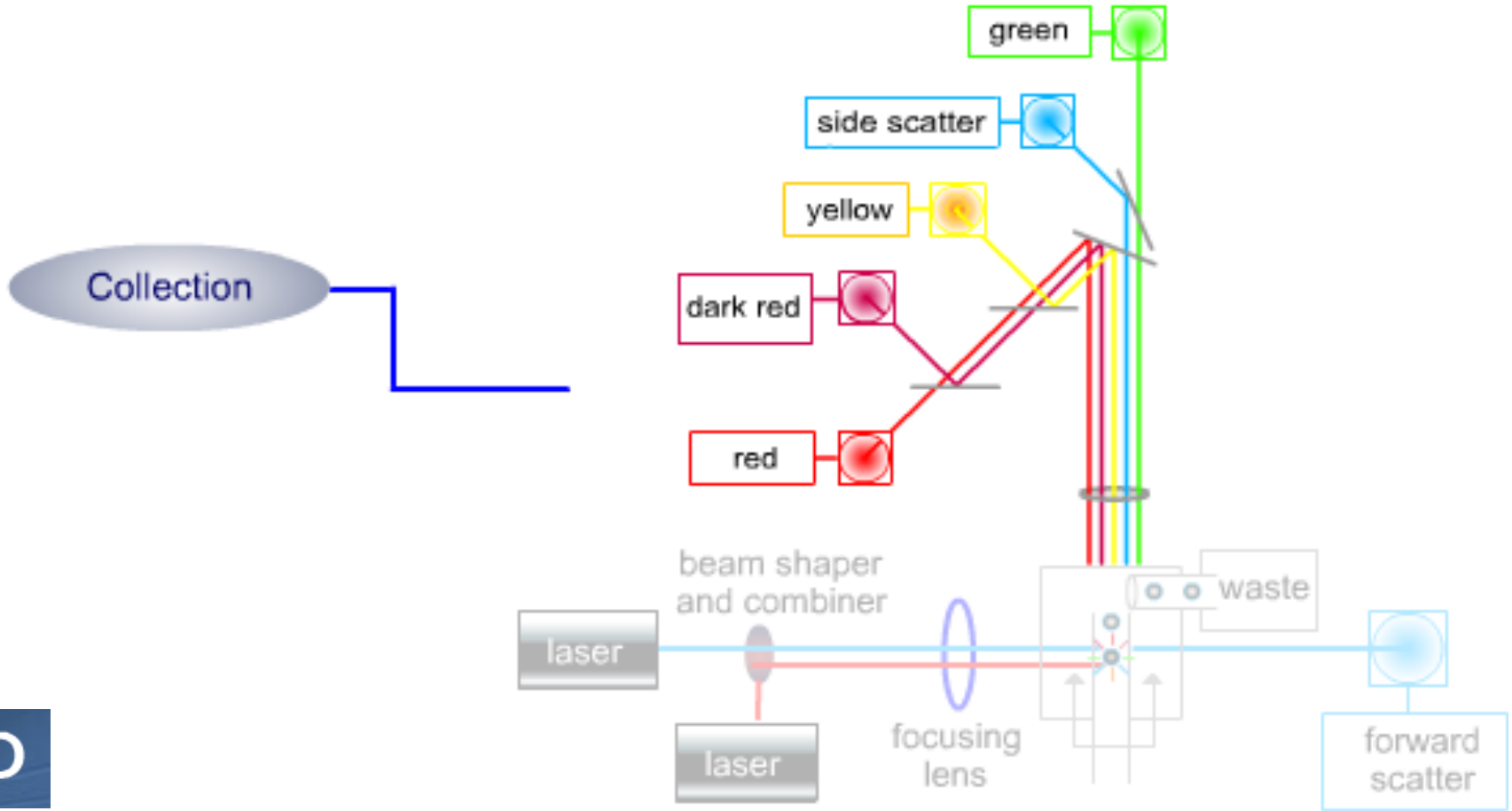


givan/dartmou

Collection Optics

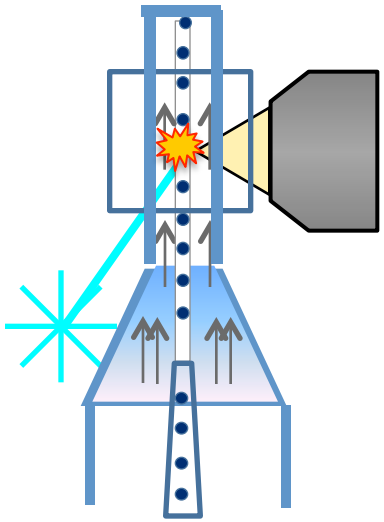
Lenses, mirrors and filters

separate wavelengths and direct to detectors

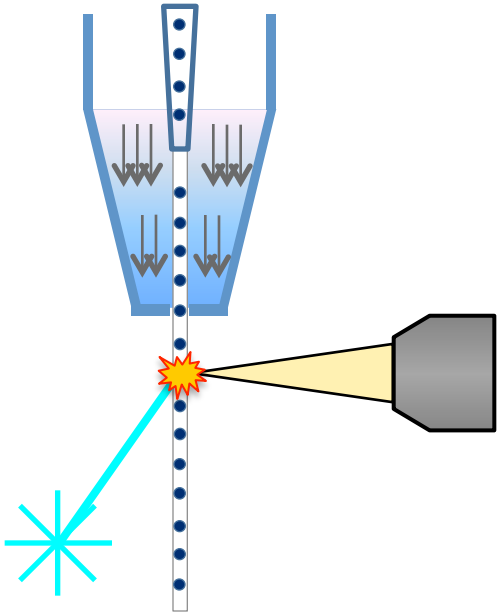


Fluorescent light emission is first collected through a lens

flow cell



Stream-in-air



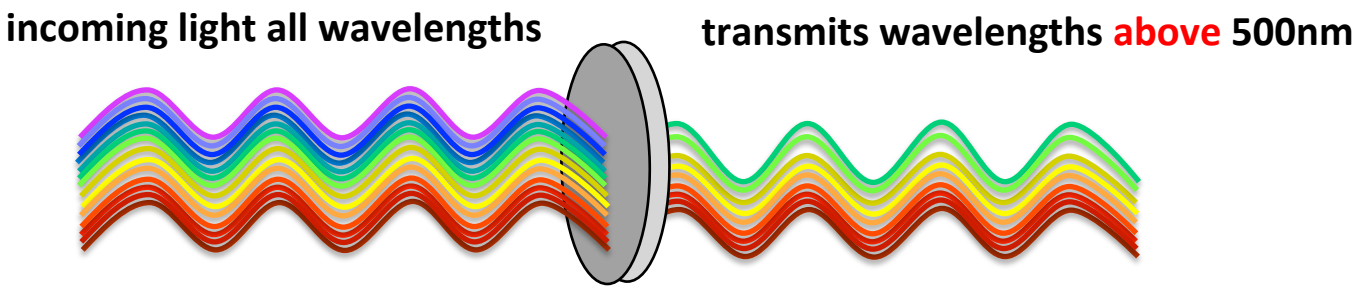
Here the lenses are shown at 90° to the axis of the lasers

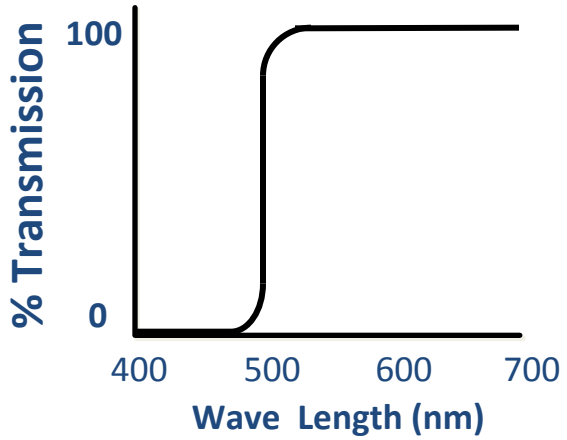
After collection by the lens, the emitted light then

- passes through optical mirrors and filters
- which separate the different wavelengths
- and direct them to the right detectors

Optical Filters: Long Pass

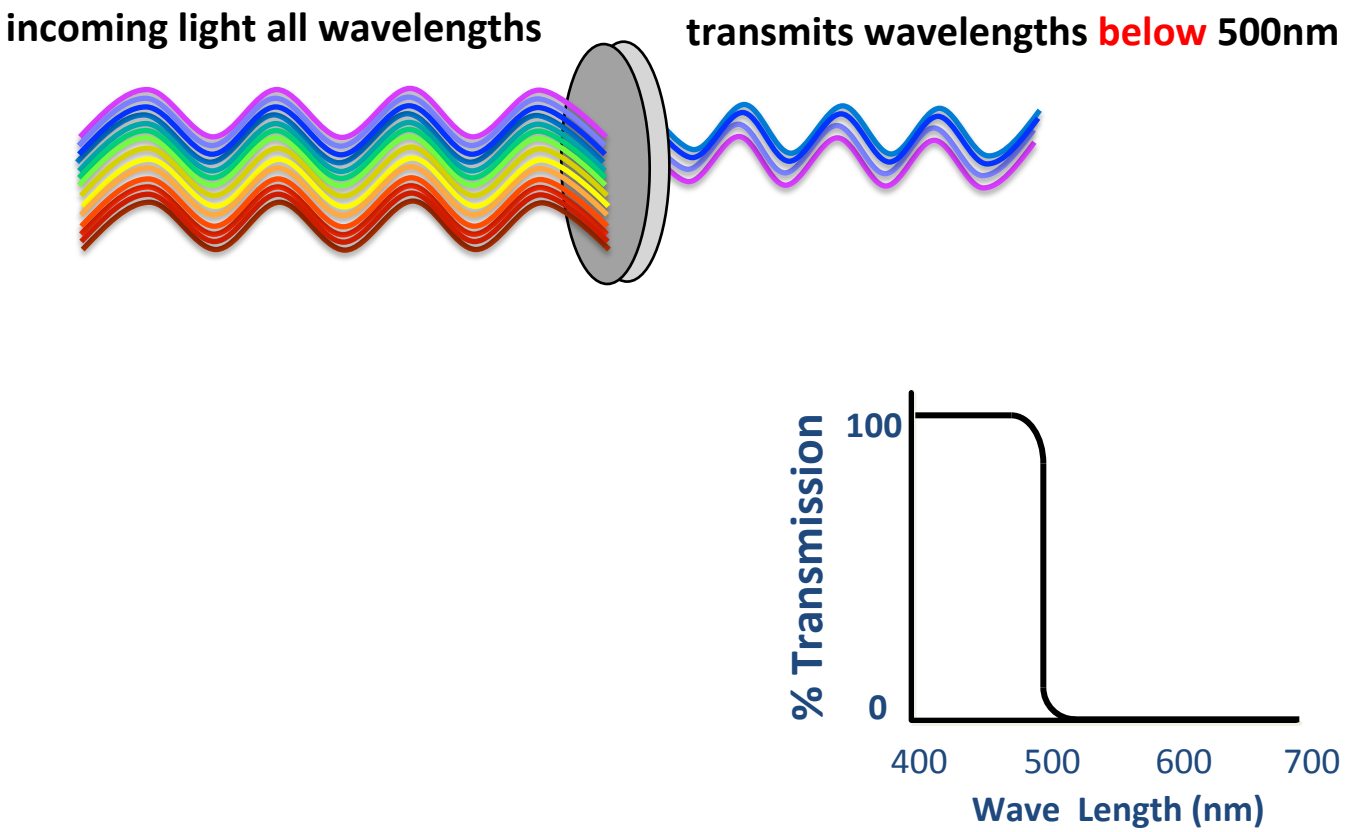
Long Pass LP500





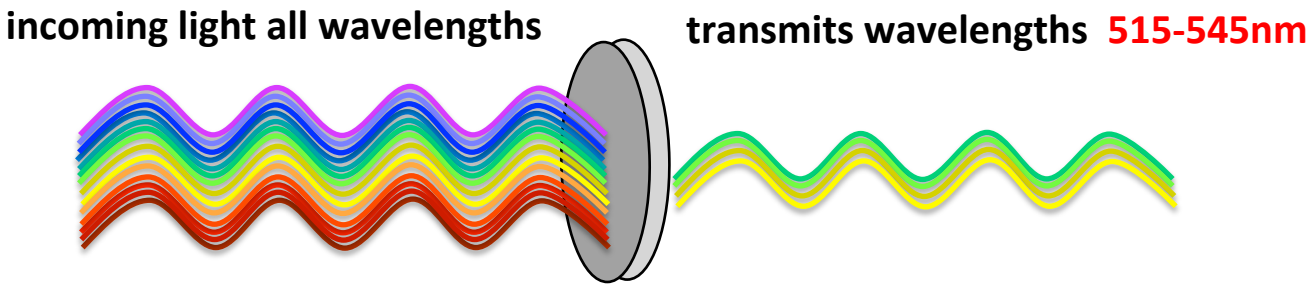
Optical Filters: Short Pass

Short Pass Filter SP500



Optical Filters: Band Pass

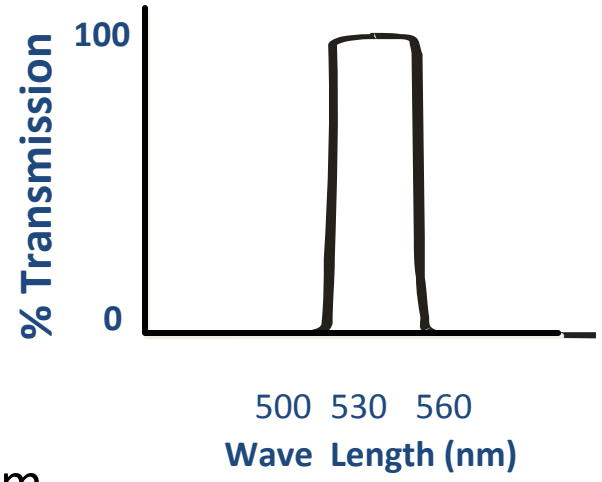
Band Pass Filter BP530/30



Bandpass → BP 530/30 nm

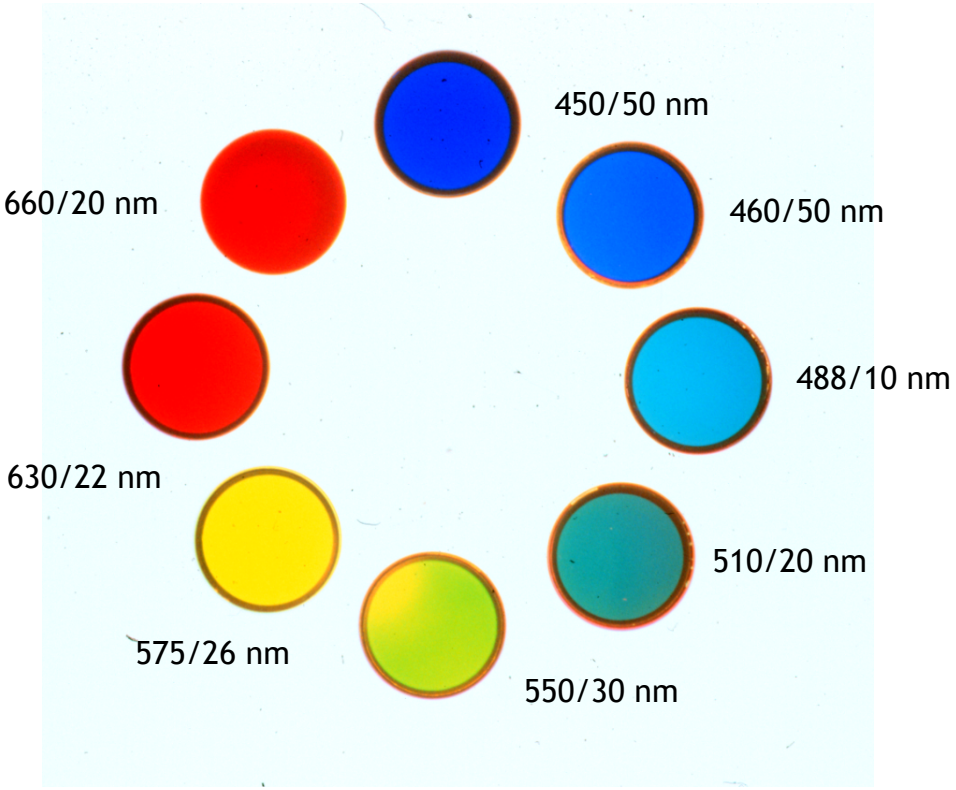
The first number refers to the center wavelength of the filter.

The second number refers to the size of the filter window.



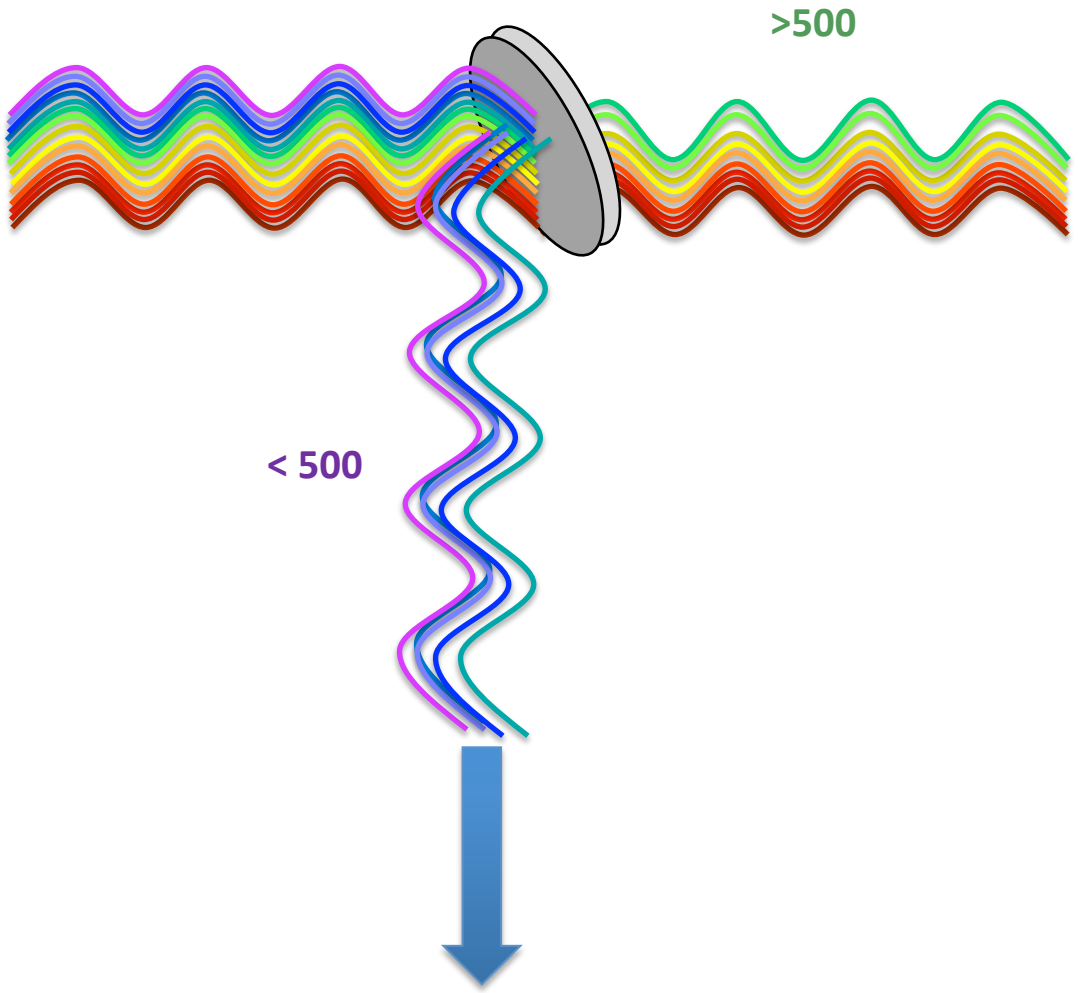
This means it transmits 530+/-15 or 515-545 nm

A rainbow of bandpass filters are available in a wide range of wavelengths

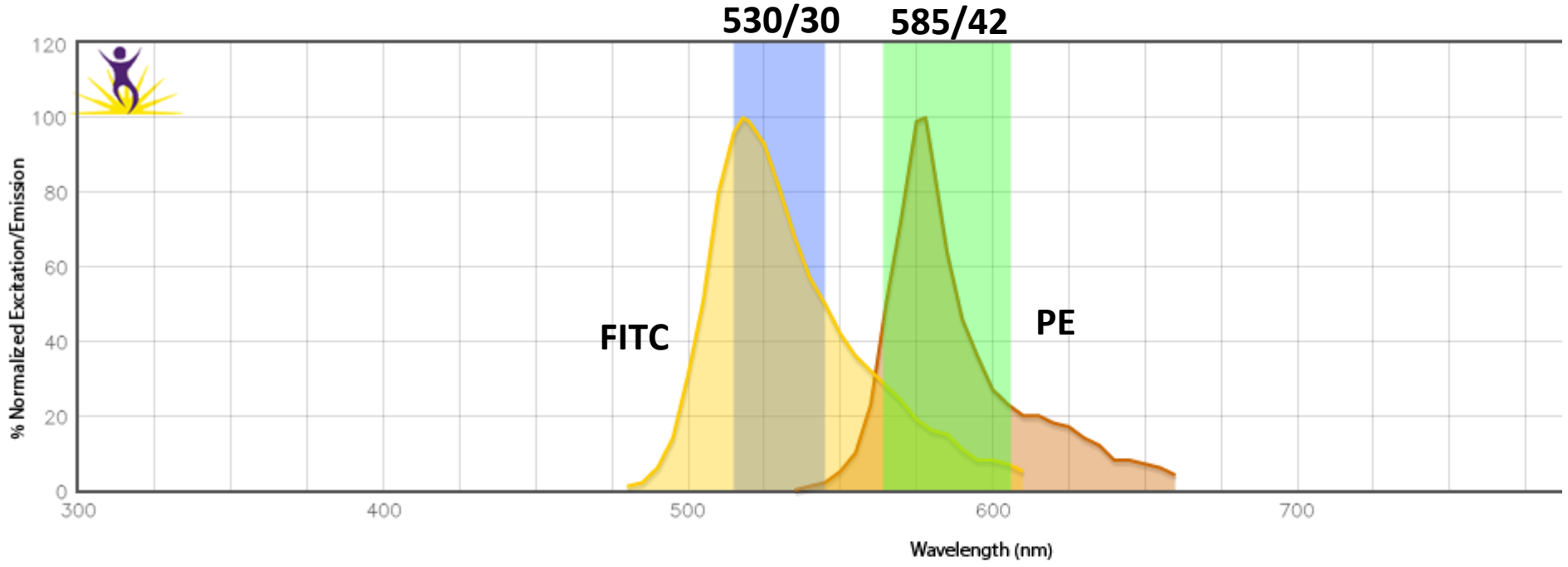


Dichroics: filters and mirrors

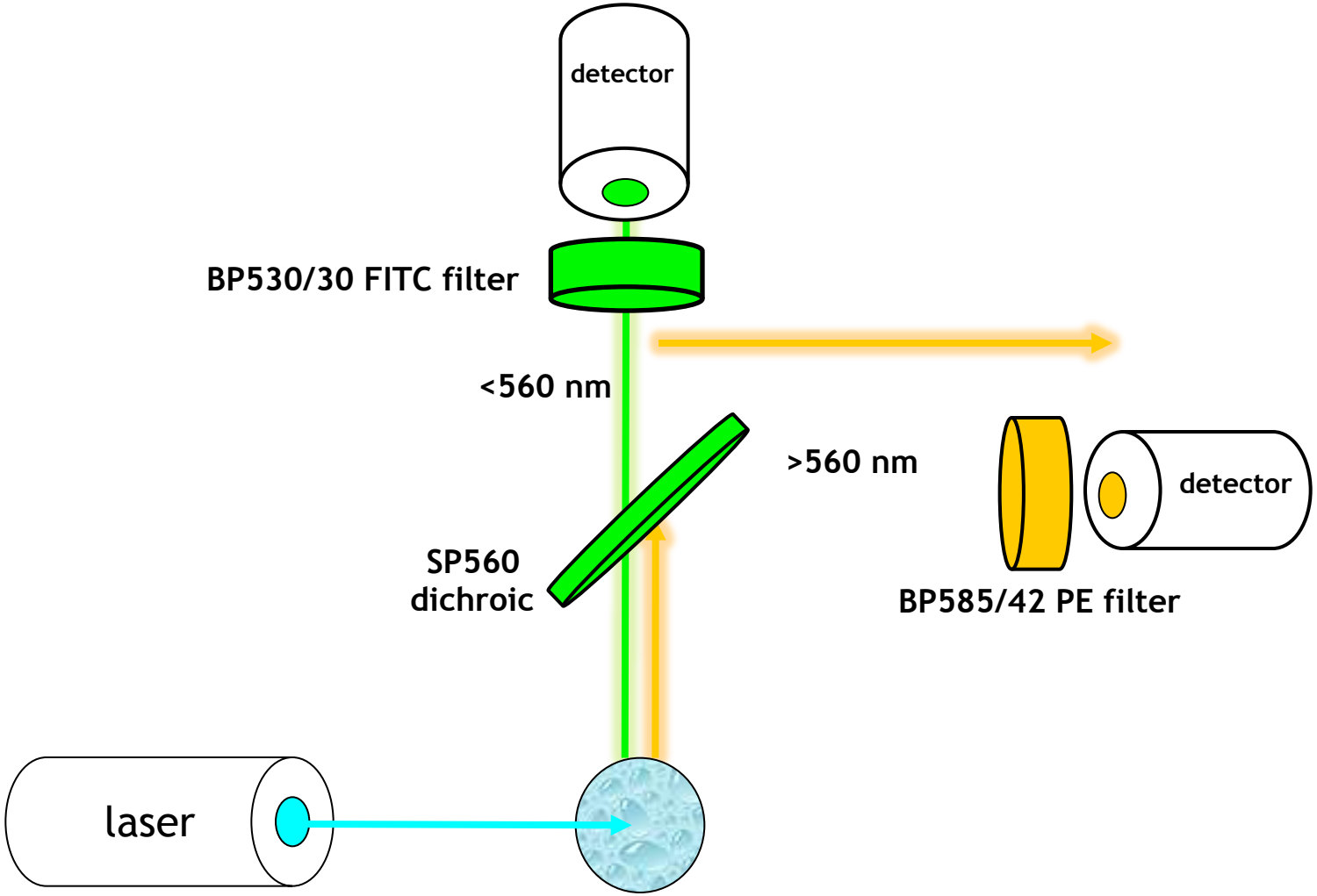
LP500 filter is angled to use as a dichroic mirror



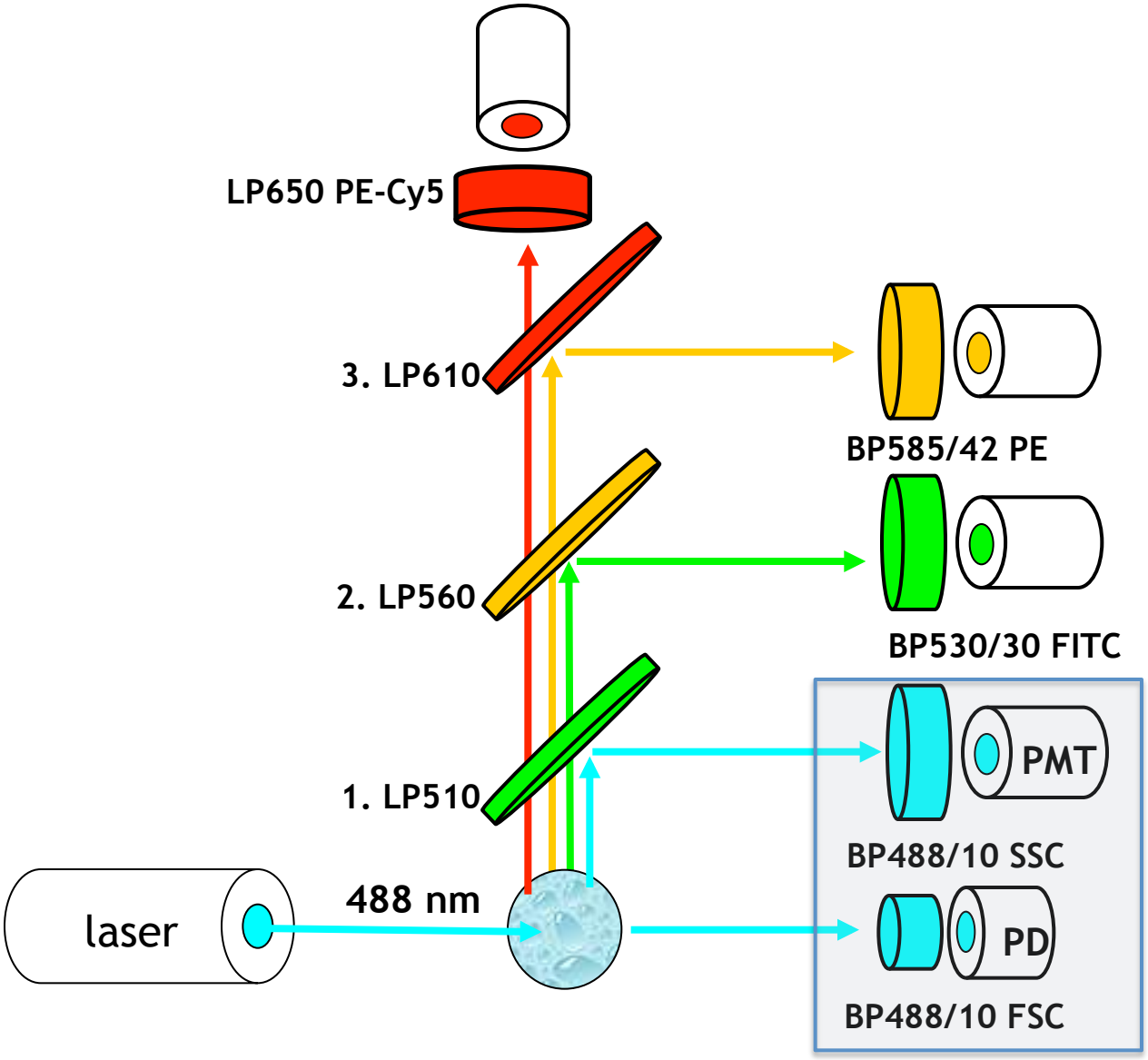
Know the emission spectra of your fluorochromes and which filters are best adapted

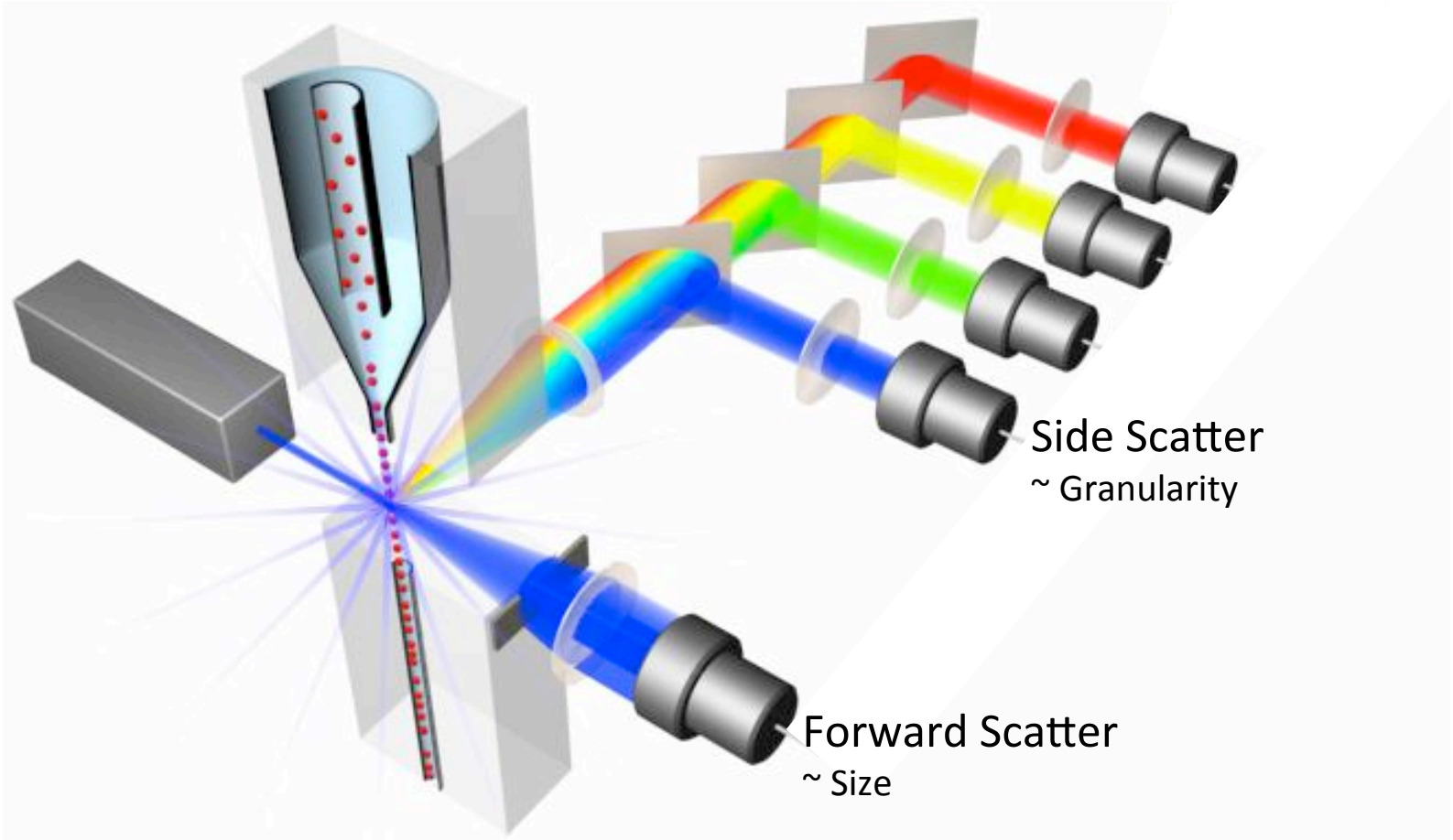


2 color fluorescence detection
FITC and PE



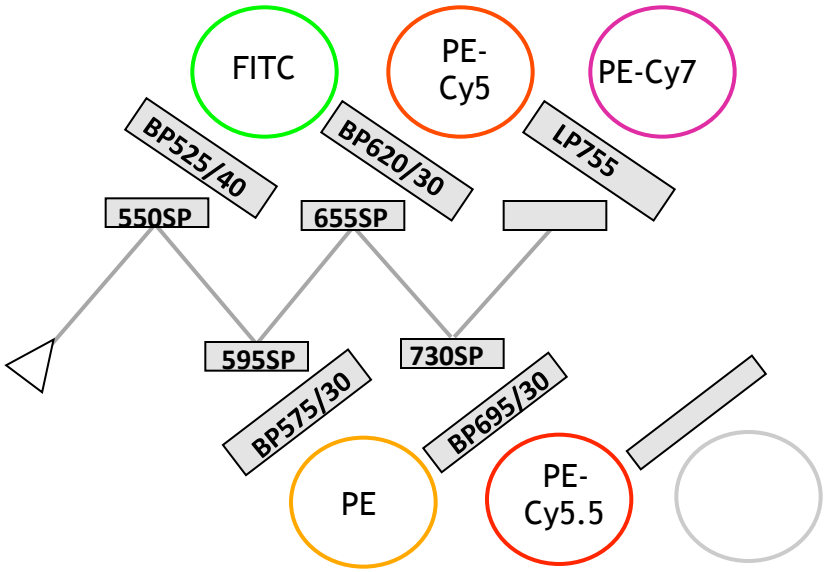
3 color fluorescence plus scatter detection



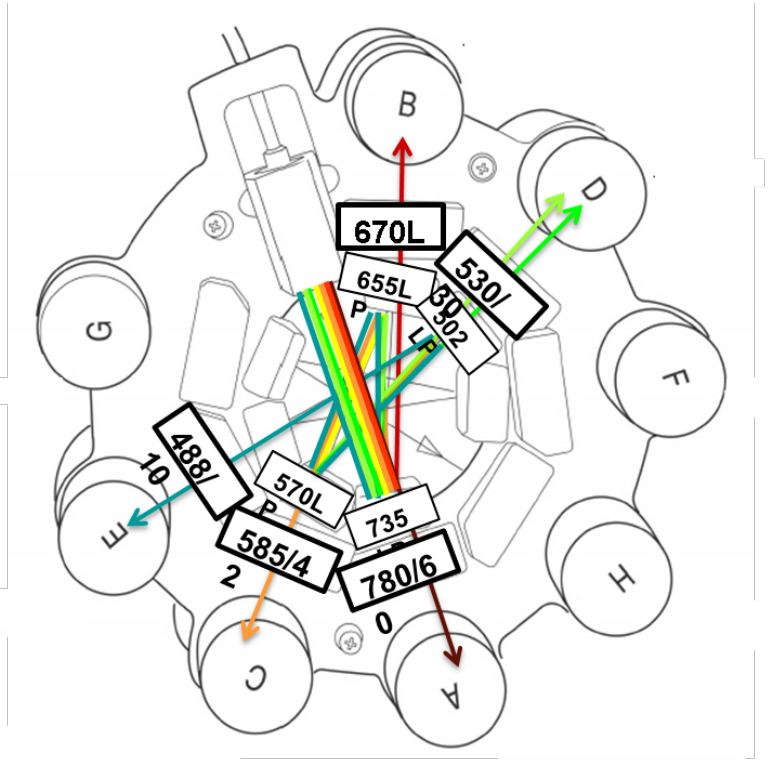

Side Scatter
~ Granularity
Forward Scatter
~ Size

Some Typical Optical Schemes

Linear array

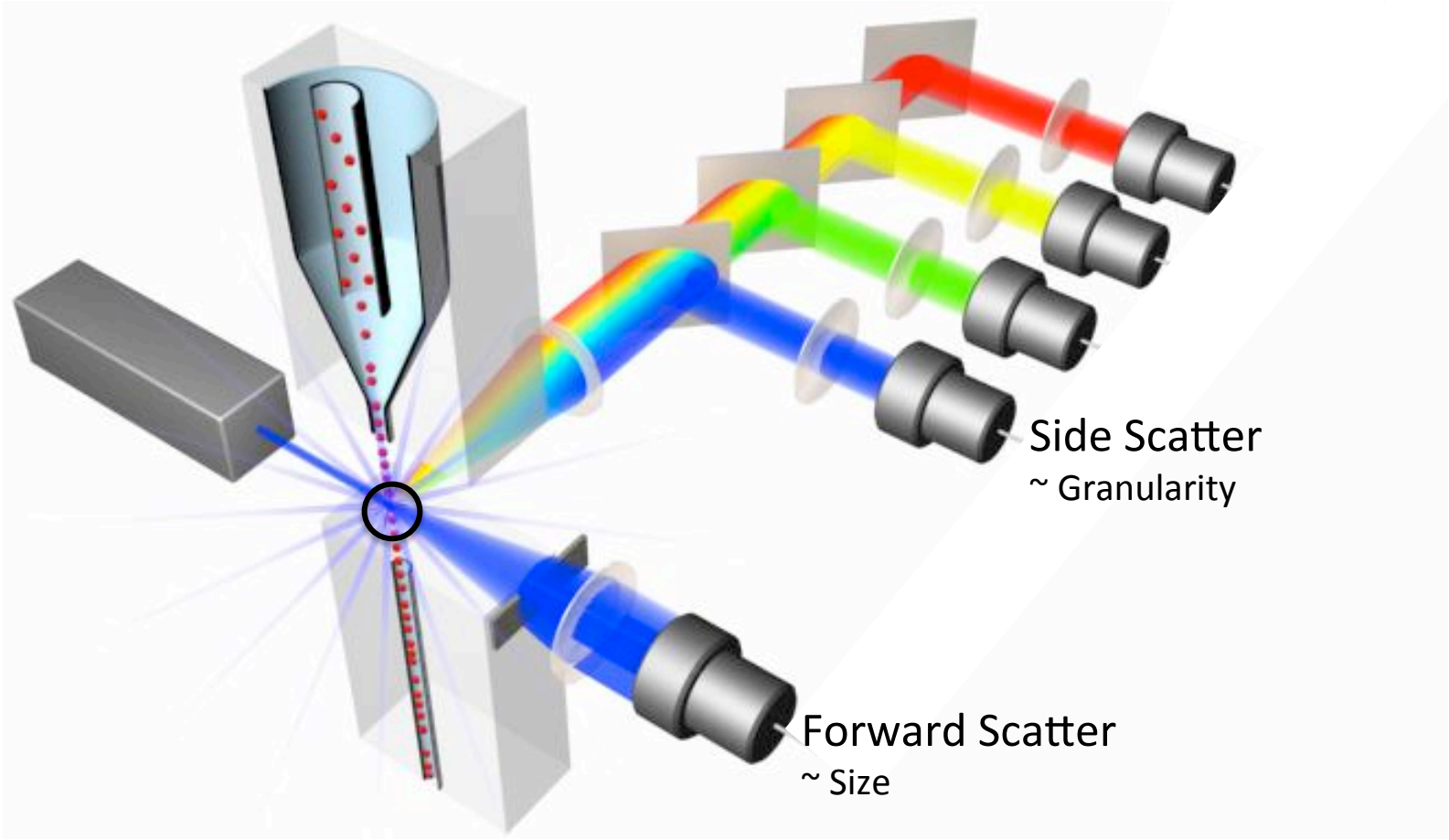


Octagon

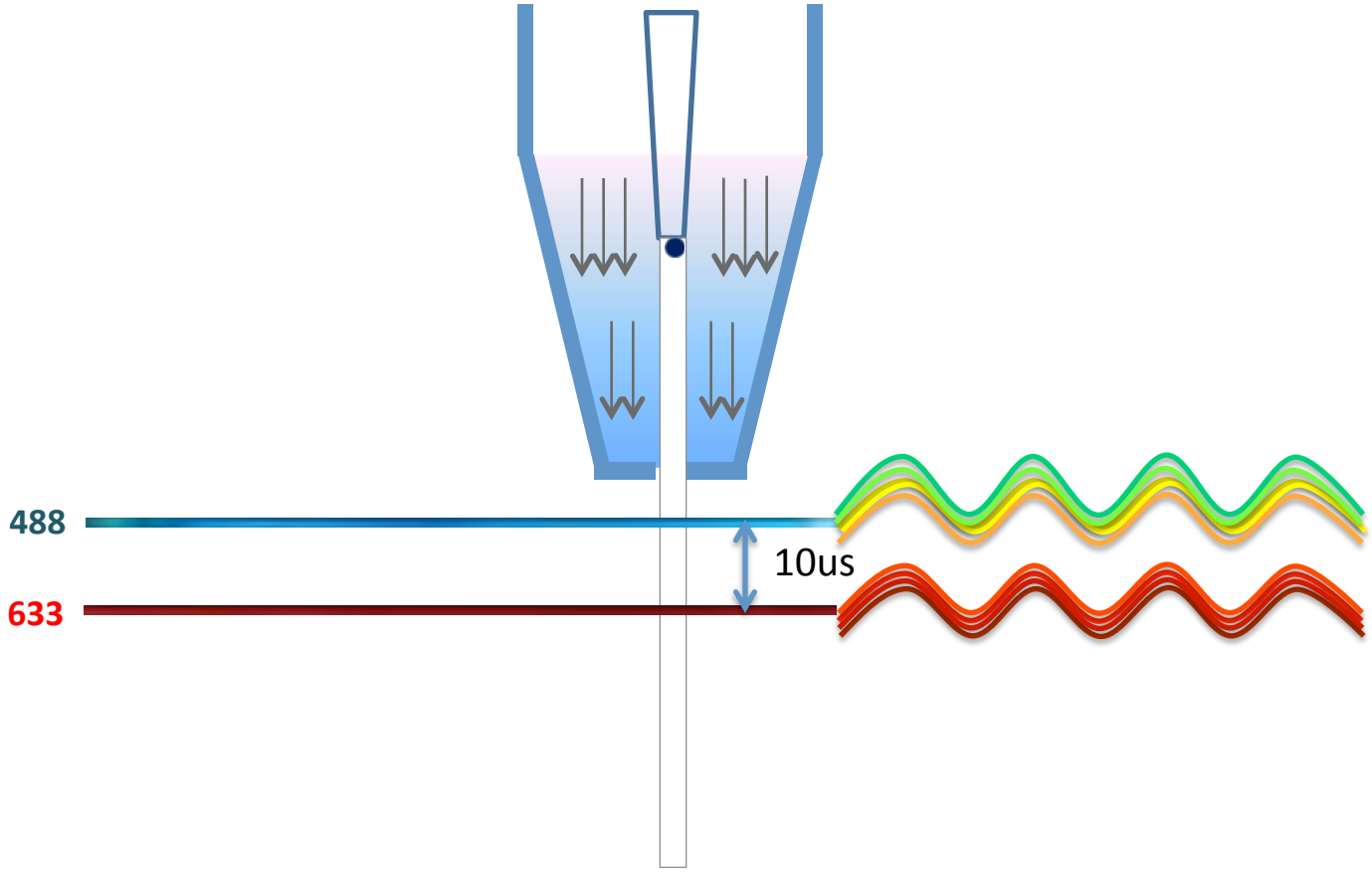


SIngle laser

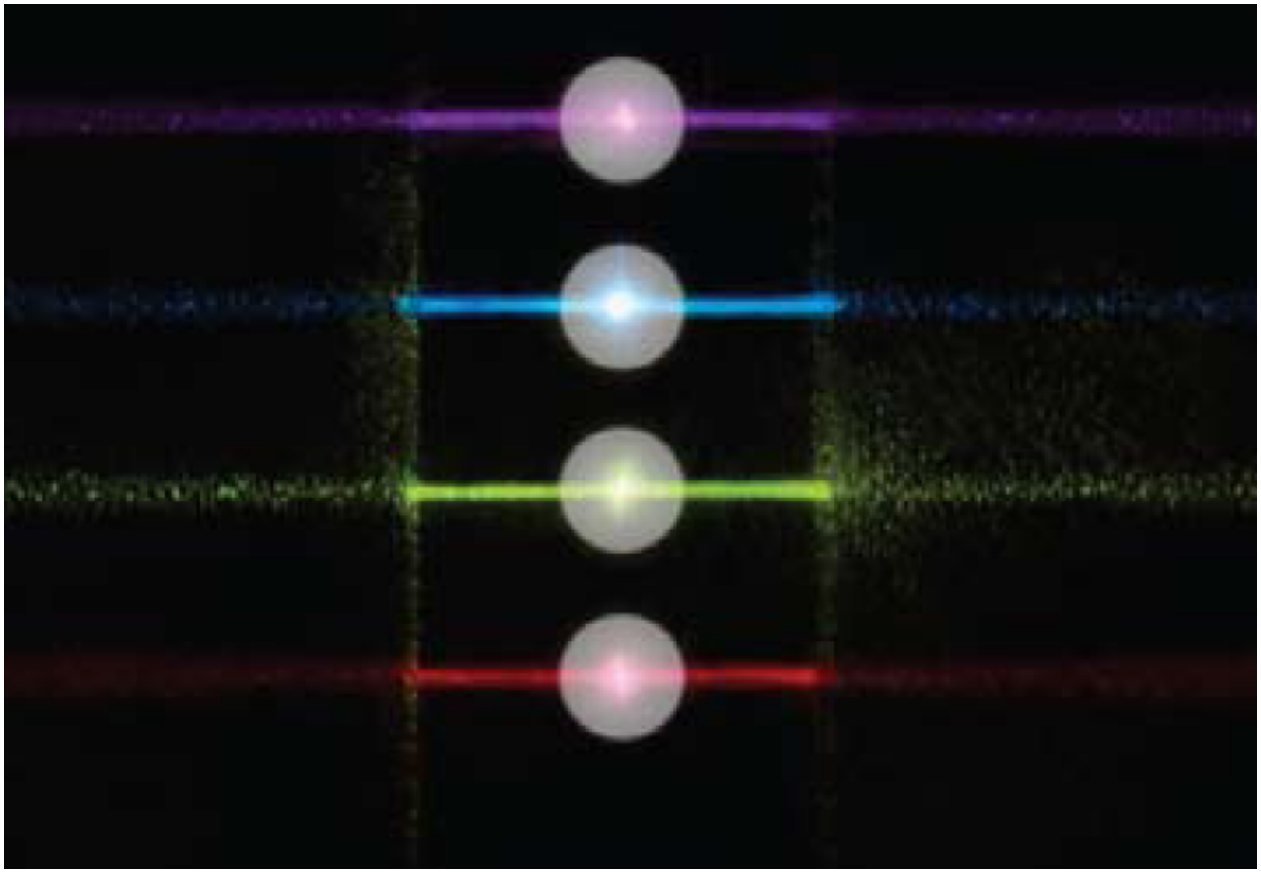
So far, we have been looking at the excitation and emission from only one laser

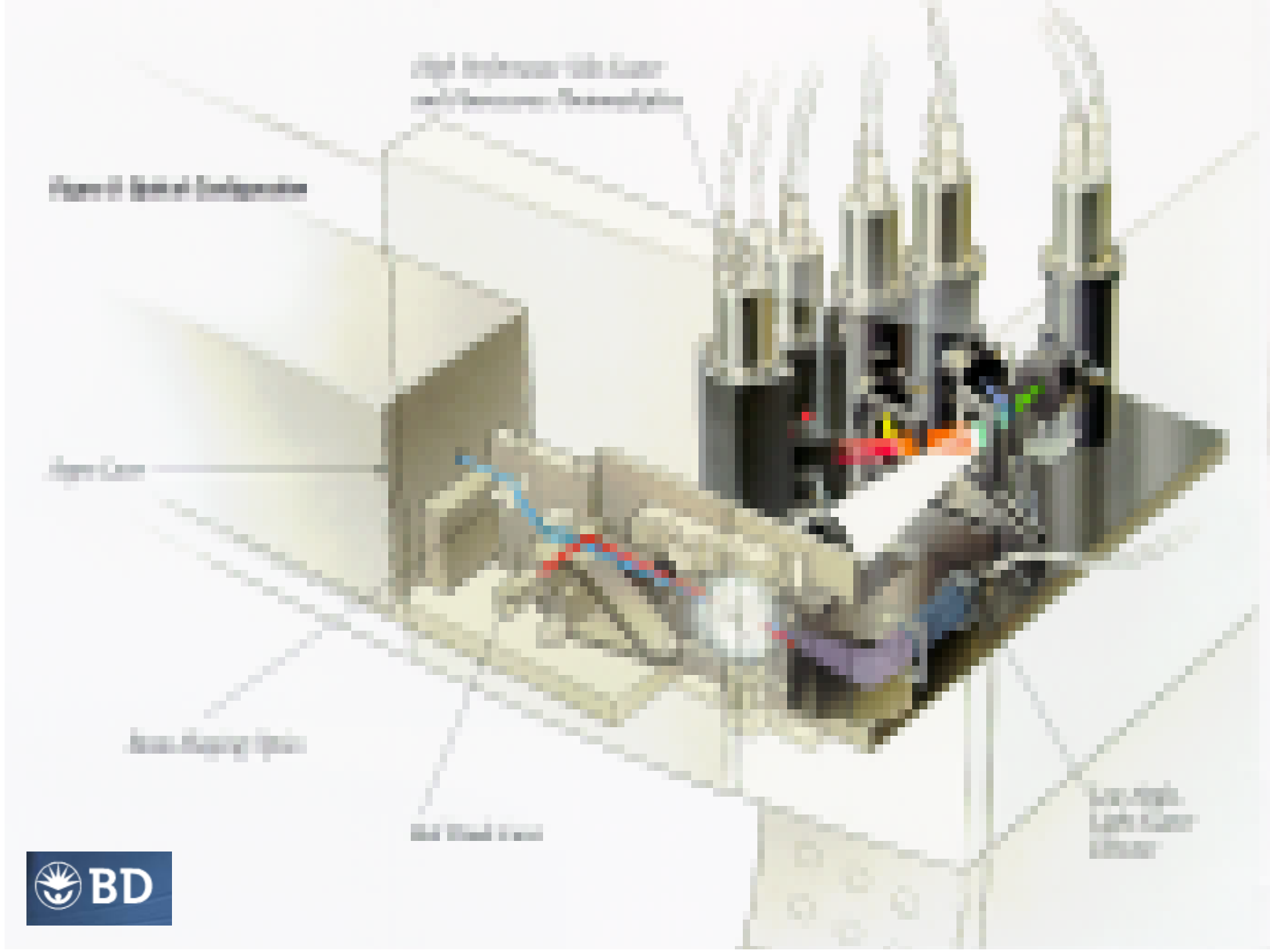


What happens when there are 2 lasers? separation in space and time

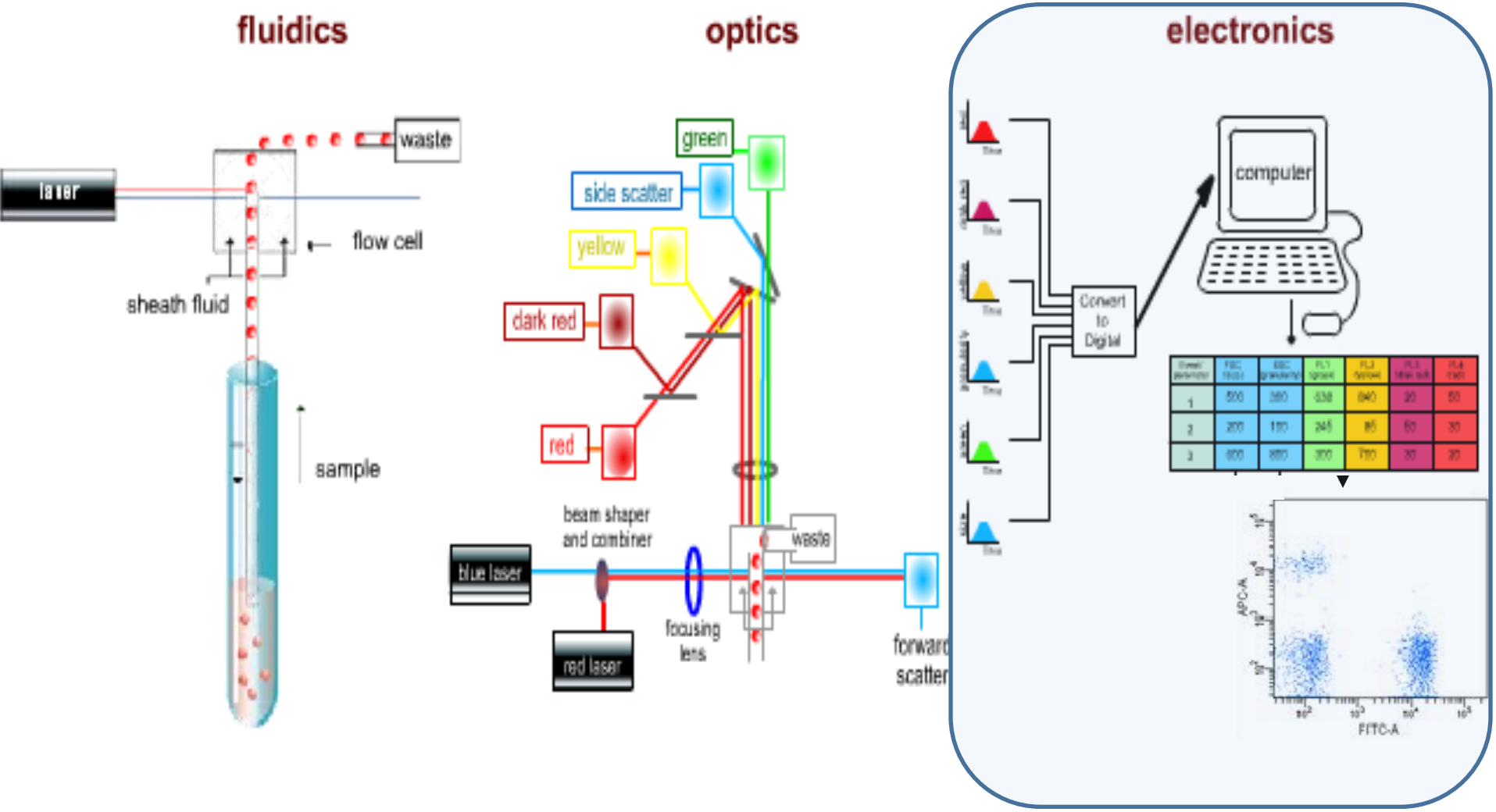


Most cytometers have 3 to 5 lasers

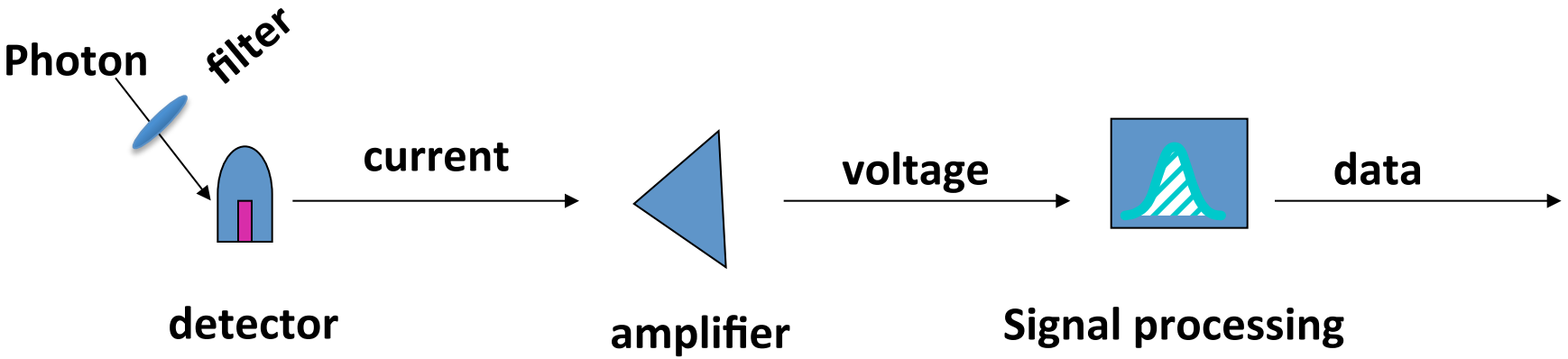




Flow Cytometer Elements



Electronics overview



Light detectors transform light photons into electrical current

voltage pulses proportional to each fluoresence emission are generated

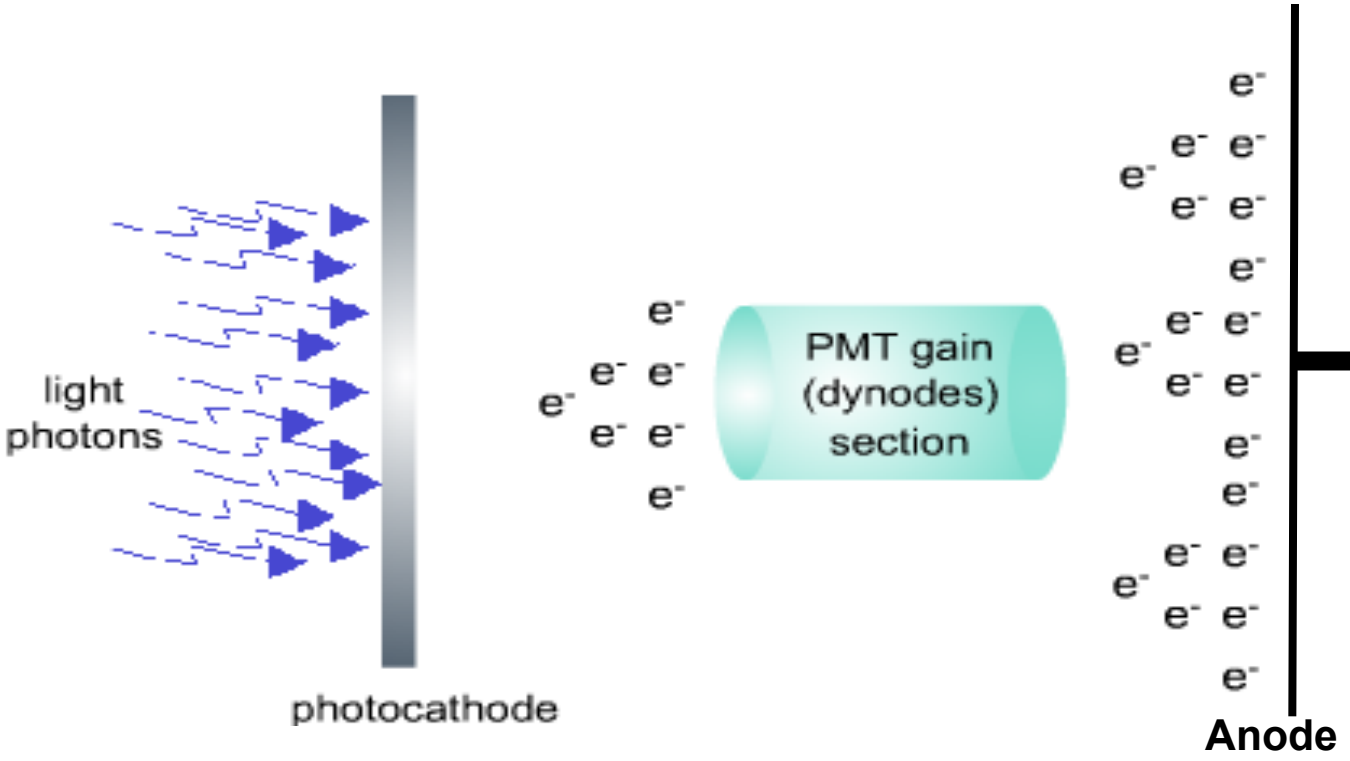
then digitalized To a numerical value

Photodetectors

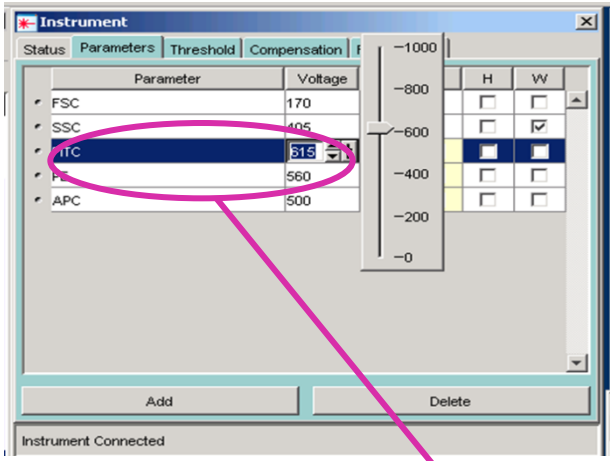
- Photodetectors transform light into electrical current
- types of photodetectors used in cytometers
    - Photodiodes:
        - Forward scatter (used for strong light signals)
        - New avalanche photodiodes APD (Cytoflex)
    - Photomultiplier tubes (PMT): used for weak light signals
        - Side scatter and all fluorescence parameters

Light Detectors

Photomultiplier Tube (PMT)



Changing the PMT voltage



- Changing the voltage applied to the dynode chain increases or decreases output signal (current) from the PMT

- This is done using the PMT voltage control on the software

- 10^3 to 10^8 electrons may reach the anode for every electron that left the cathode, depending on the voltage applied

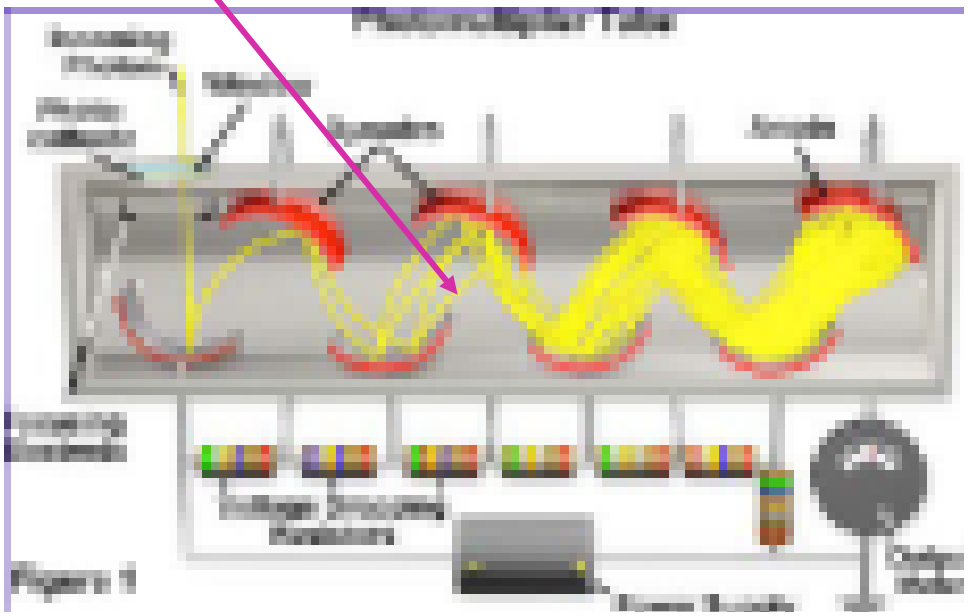
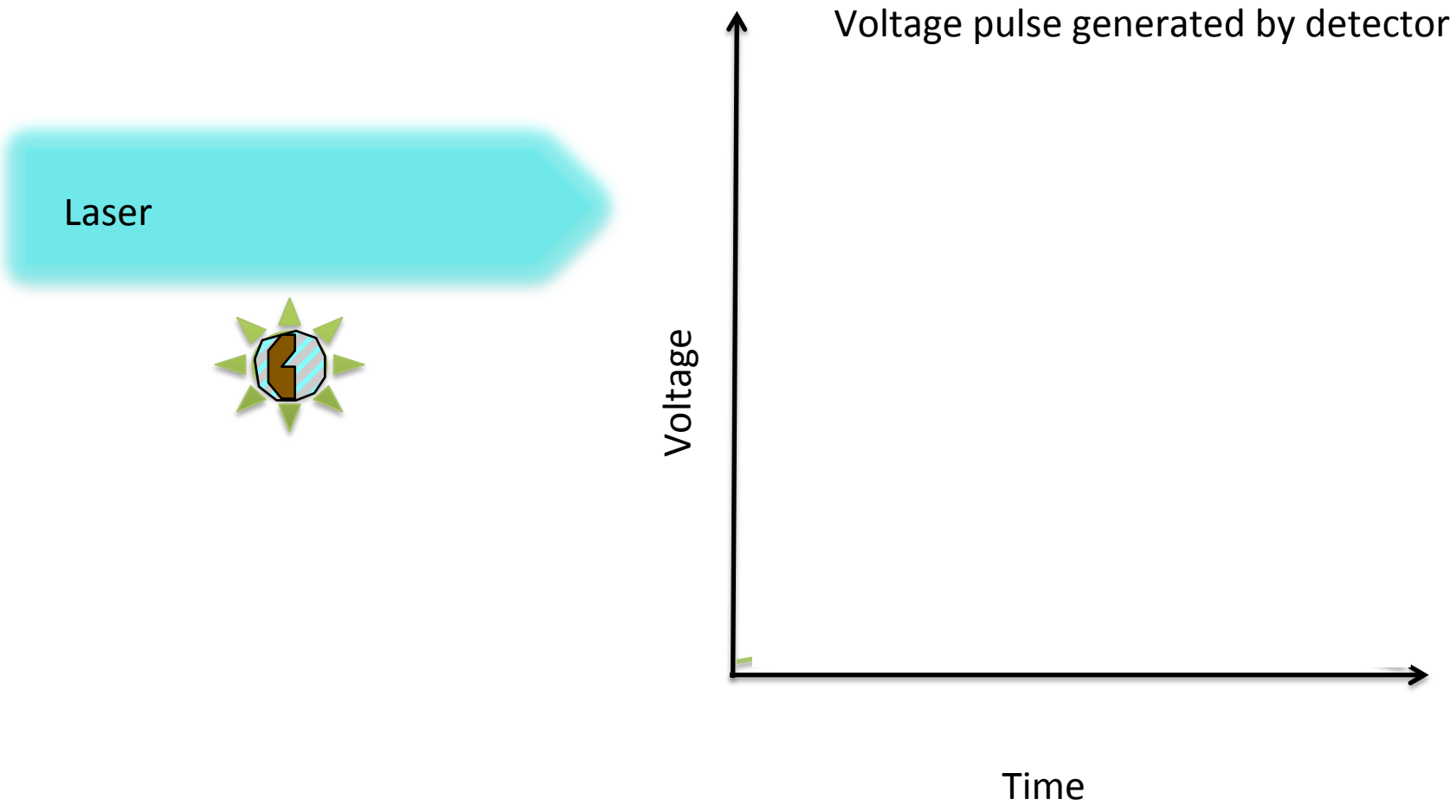


Diagram from Dakocytomation

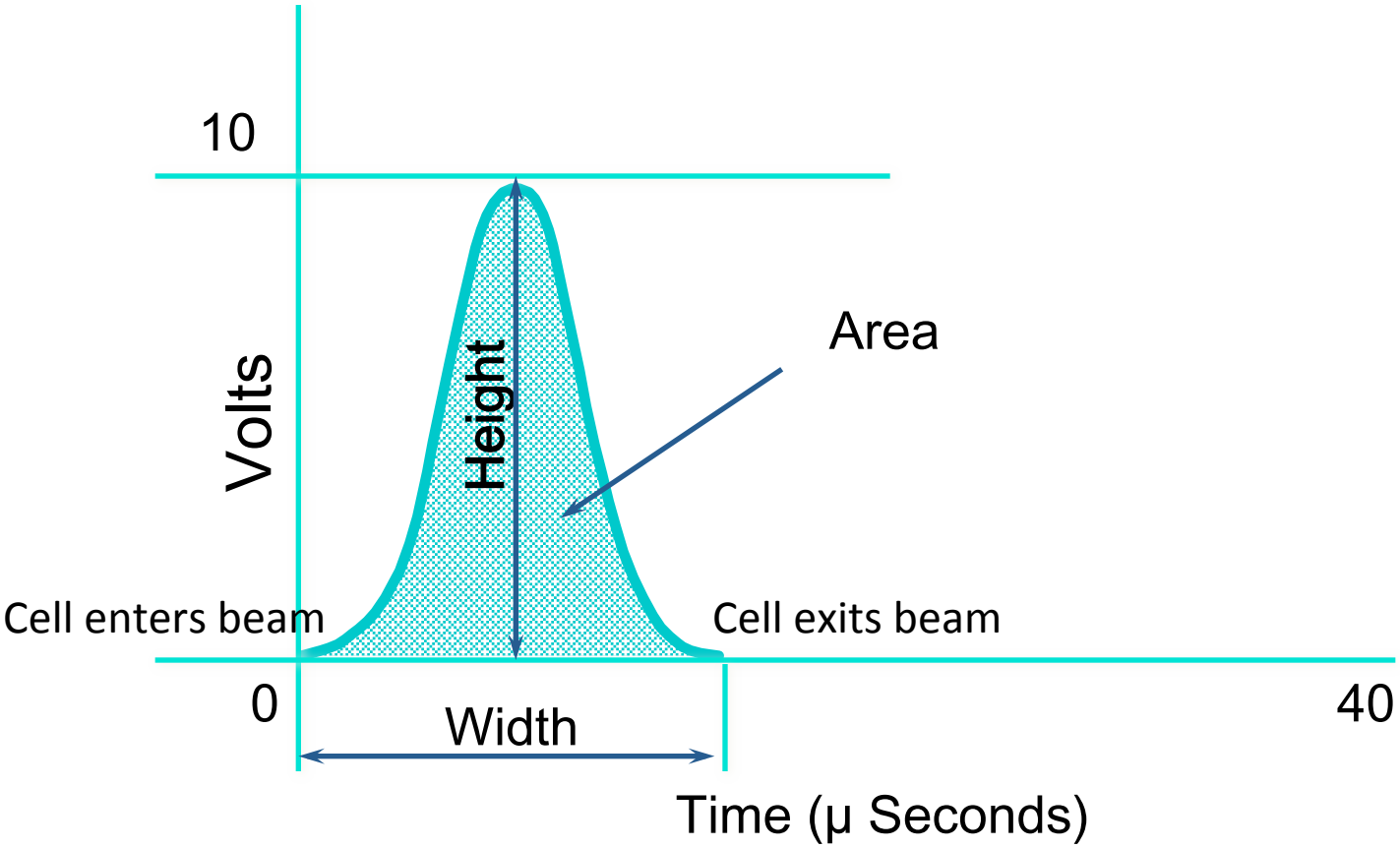
How is a pulse/signal created on a Flow Cytometer ?



Courtesy of Grace Chojnowski

Signal Processing

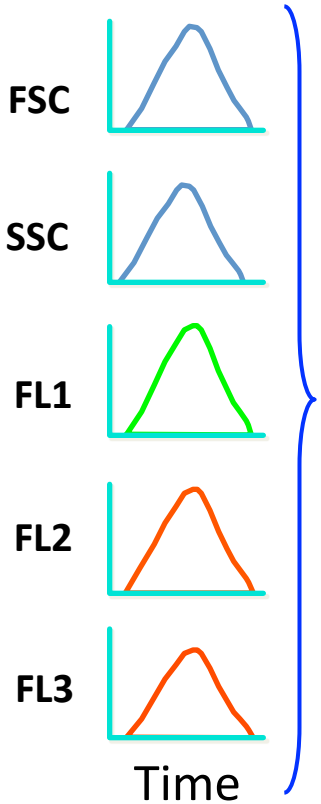
- The signal processors quantify the voltage pulses
- They generate a numerical channel value for pulse height, area and width



Digitalization

The pulse size numerical values are recorded as channel numbers
The data is saved as a list mode (.fcs) file which records all values for each event

Voltage Pulses from all detectors



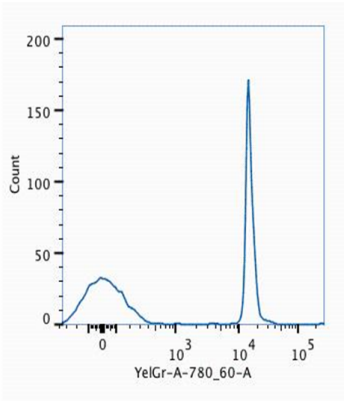
Pulse processing

Data Acquisition - Listmode

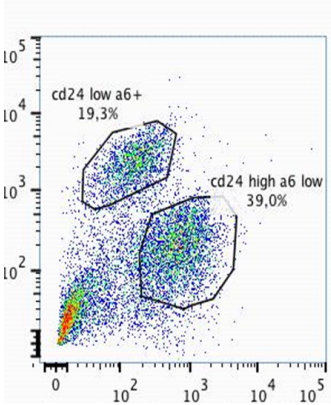
Event	*Param1 FS*	*Param2 SS*	*Param3 FITC*	*Param4 PE*
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

[RFM]

Single parameter frequency histogram



Dual parameter dotplot



List mode file

A list mode (.fcs) files contains scatter and fluorescence values for each event as well as instrument settings and cytometer information.

FCS DATA FILE (TRANSLATED)

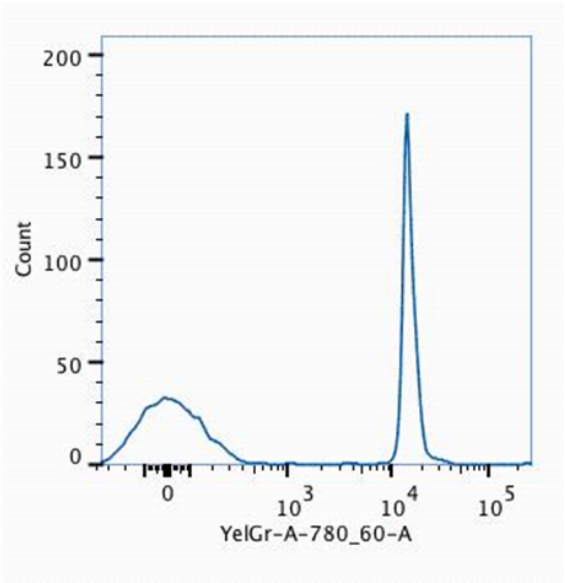
CELLS IN SEQUENCE	FSC-H	SSC-H	FL1-H	FL2-H
1	120	28	152	24
2	190	169	42	60
3	175	149	56	52
4	107	25	149	0
5	97	22	151	26
6	174	136	47	36
7	190	127	42	47
8	106	14	148	0
9	86	17	165	23
10	90	16	149	31
11	184	163	58	50
12	191	160	39	40
13	101	24	152	19
14	126	36	153	0
15	126	28	157	0
16	96	17	155	0
17	215	224	61	59
18	165	95	55	46
19	173	73	49	43
20	91	27	158	0
21	210	180	59	52
22	179	161	60	52
23	165	93	54	35
24	187	45	67	52
25	192	184	48	50
26	111	25	149	17
27	207	206	58	40

Data Acquisition - Listmode

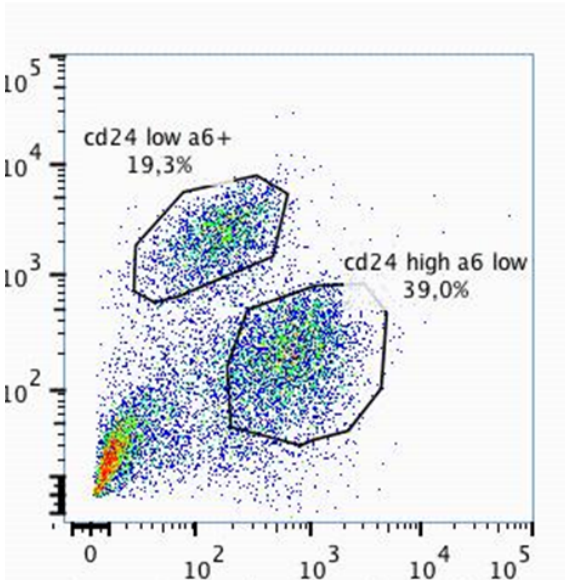
Event	*Param1 FS*	*Param2 SS*	*Param3 FITC*	*Param4 PE*
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

[RFM]

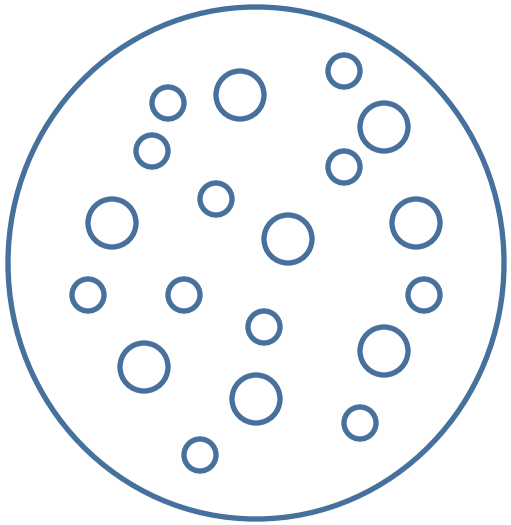
Single parameter frequency histogram

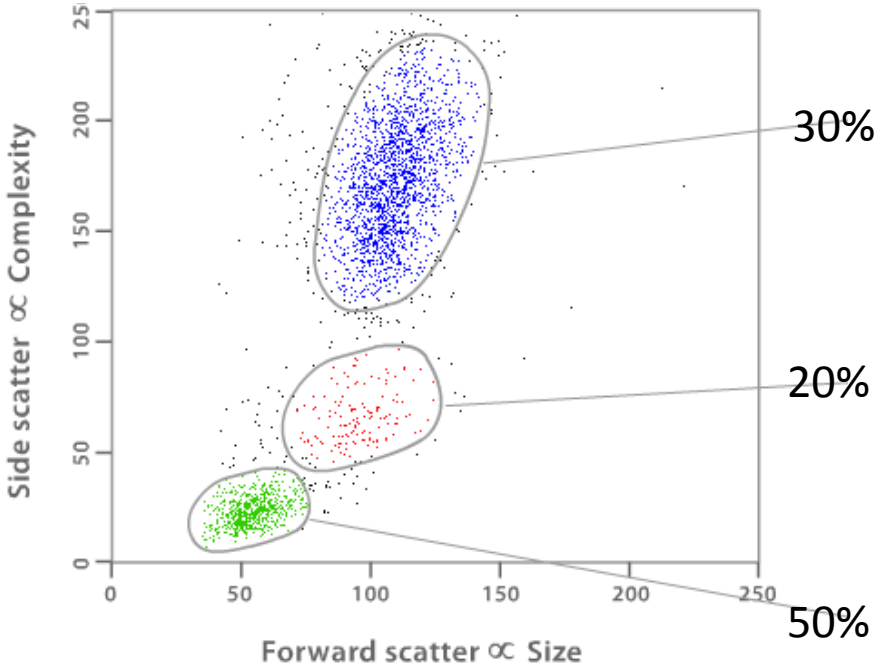


Dual parameter dotplot



So now we can answer the questions





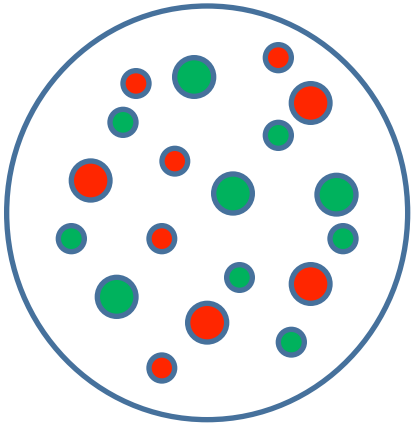
How many Small and/or Big Cells are there ?

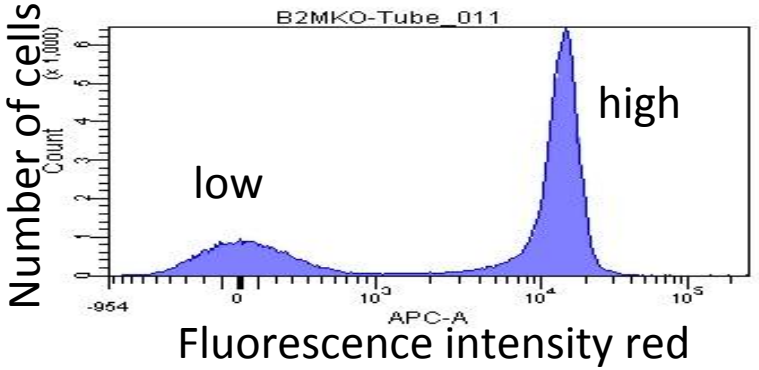
Parameter: Size

Courtesy of Dr Krishnamurthy

ncbs TIFR

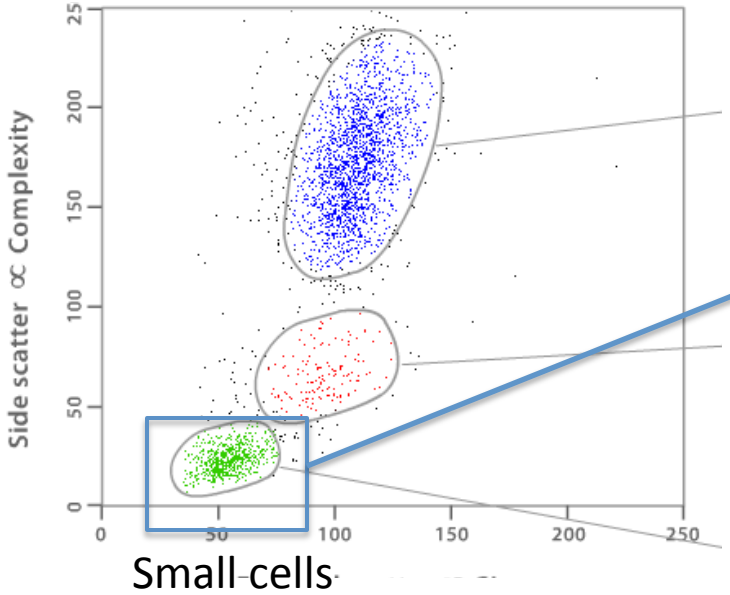
And the next questions:

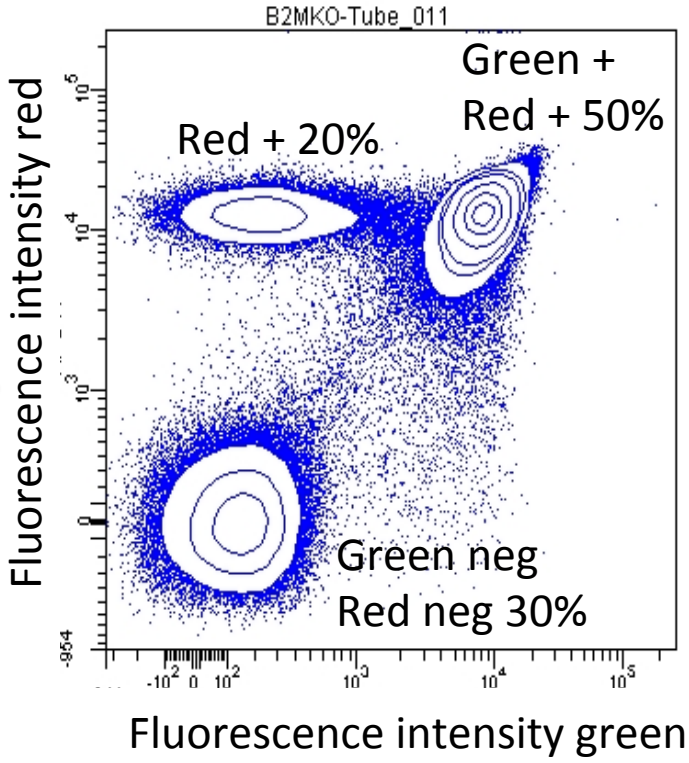




How many Small cells are Green and/or Red?

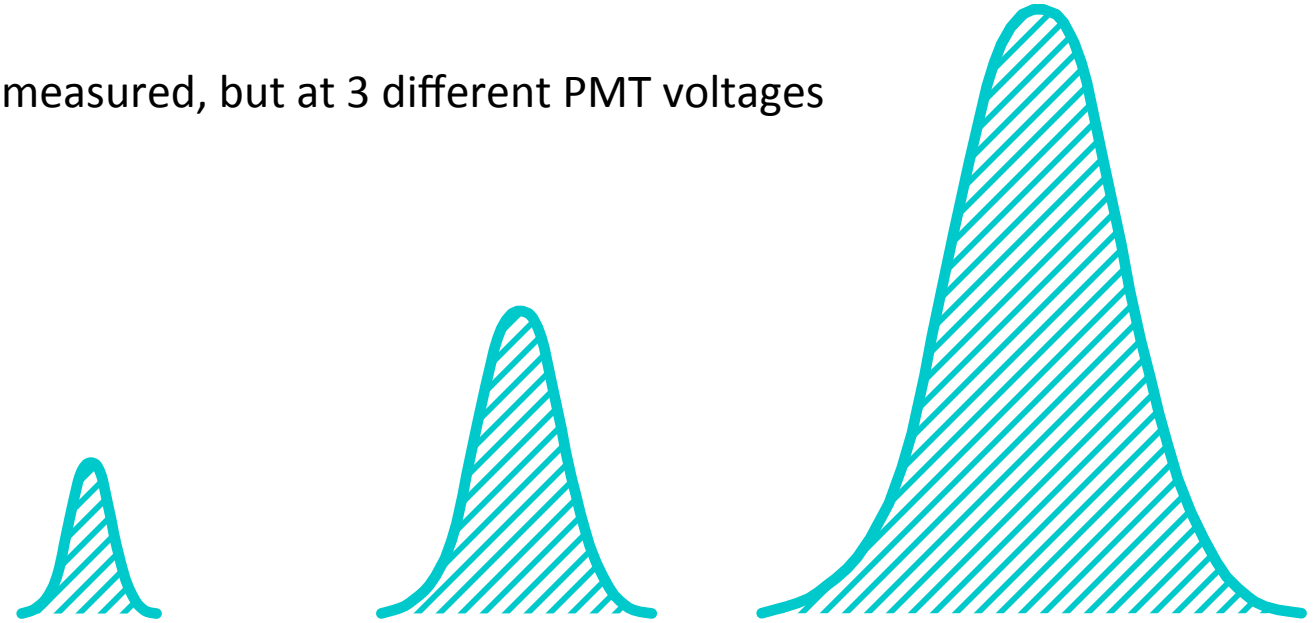
How many Big cells are Green and/or Red?



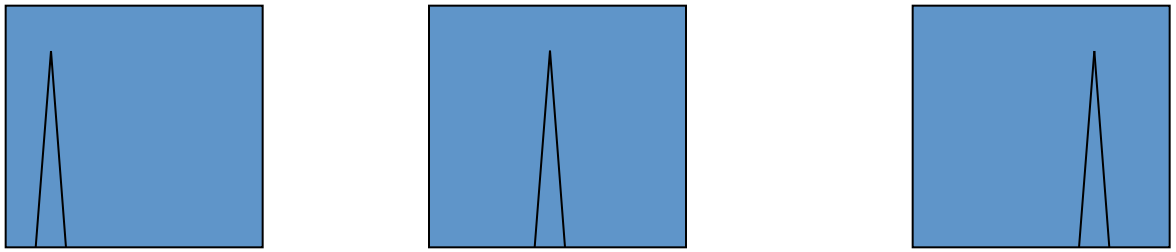


Changing the PMT voltage

The same cell is measured, but at 3 different PMT voltages



300v **400v** **500v**



Forward scatter

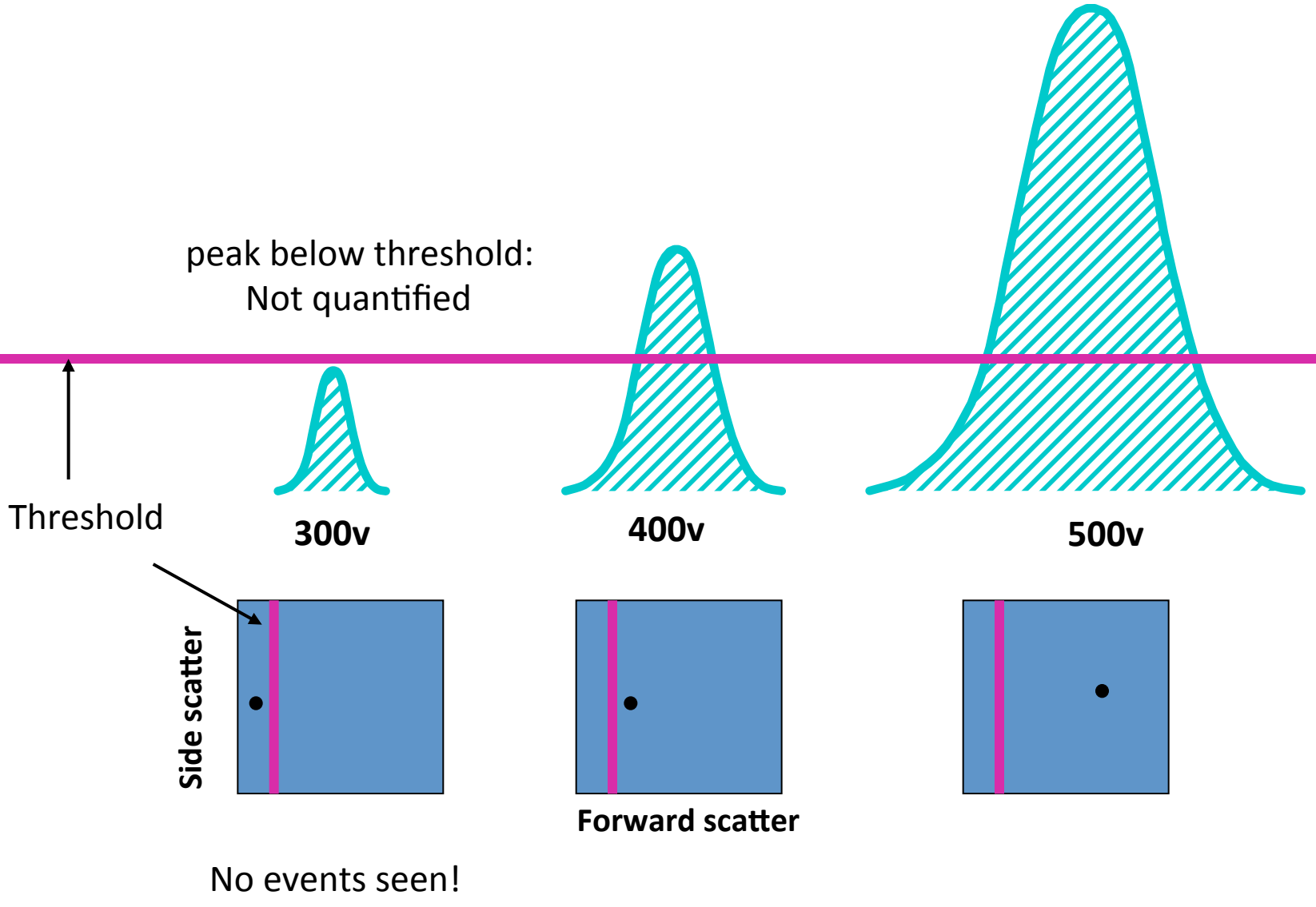
Threshold

The cytometer needs a threshold to determine what is considered an event (or cell or bead etc) and what is background or debris

Threshold: the level above which detected signals will be processed.

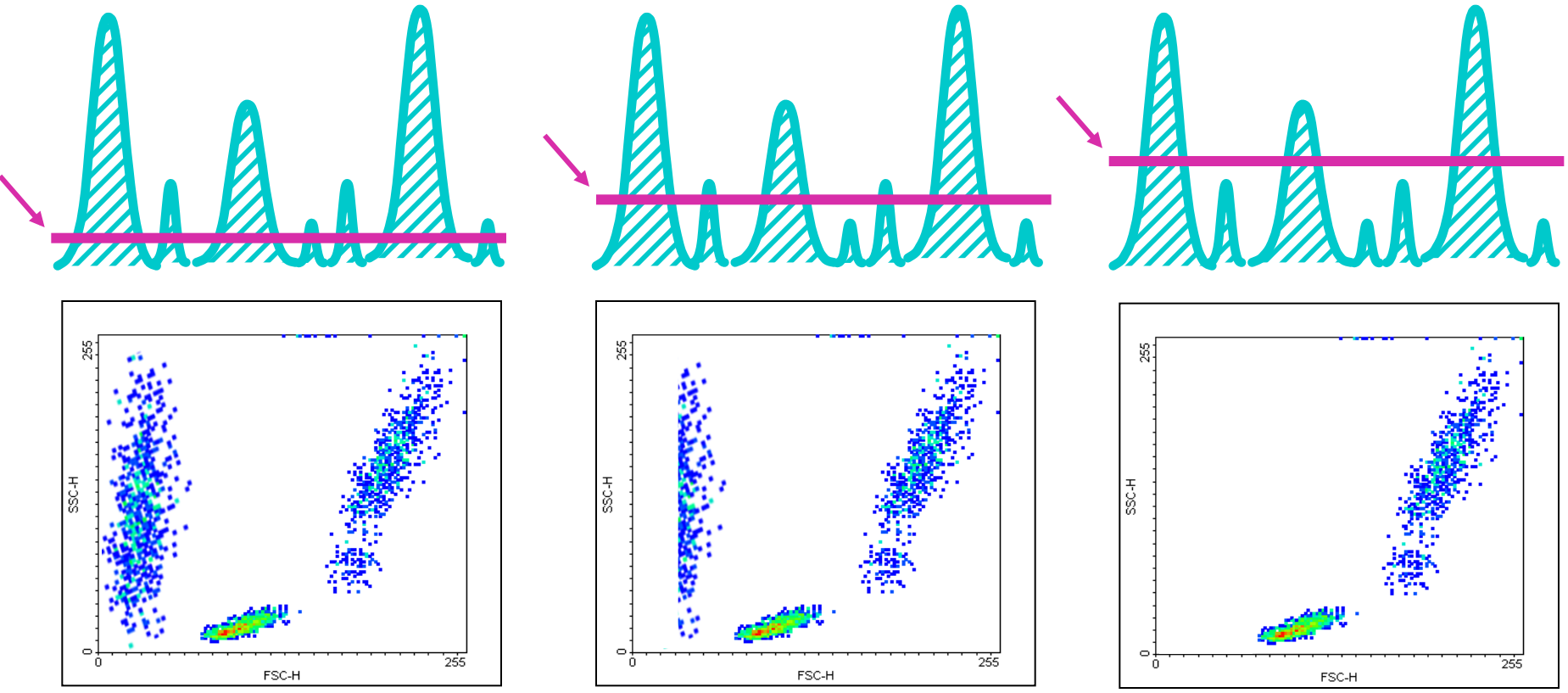
If a pulse is lower than the threshold, **it will not be seen.**
Anything below threshold is excluded from analysis.

Threshold



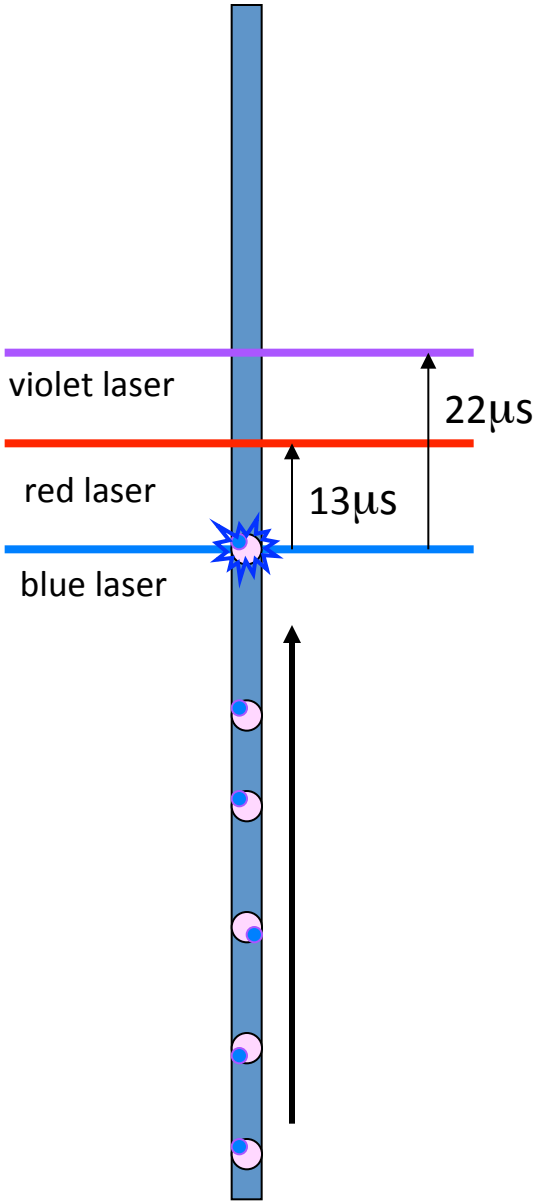
No events seen!

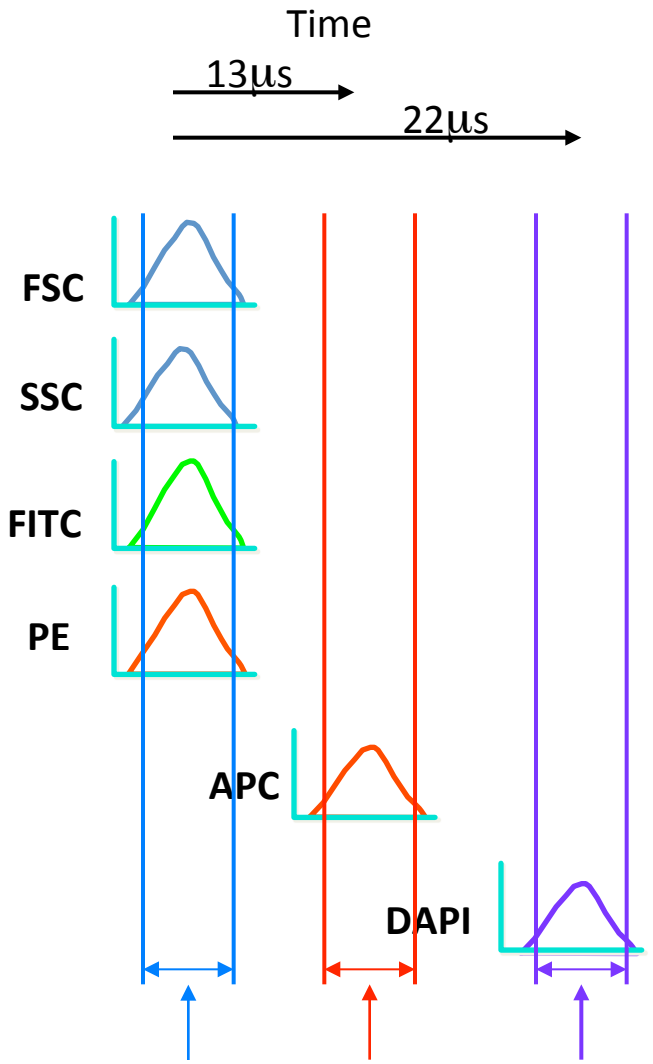
Threshold



- Increasing the threshold removes smaller pulses thus smaller events from analysis
- Events below threshold are not recorded, thus lost for good.

Laser time delay





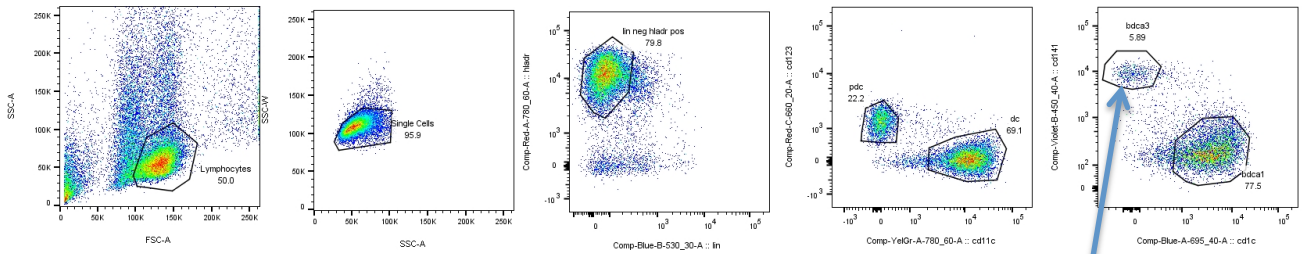
list mode (.fcs) file

	Cell # 1	Cell #2
FSC	360	450
SSC	345	375
FITC	35040	205
PE	125000	85000
APC	230	160000
DAPI	405	650

Cell Sorting

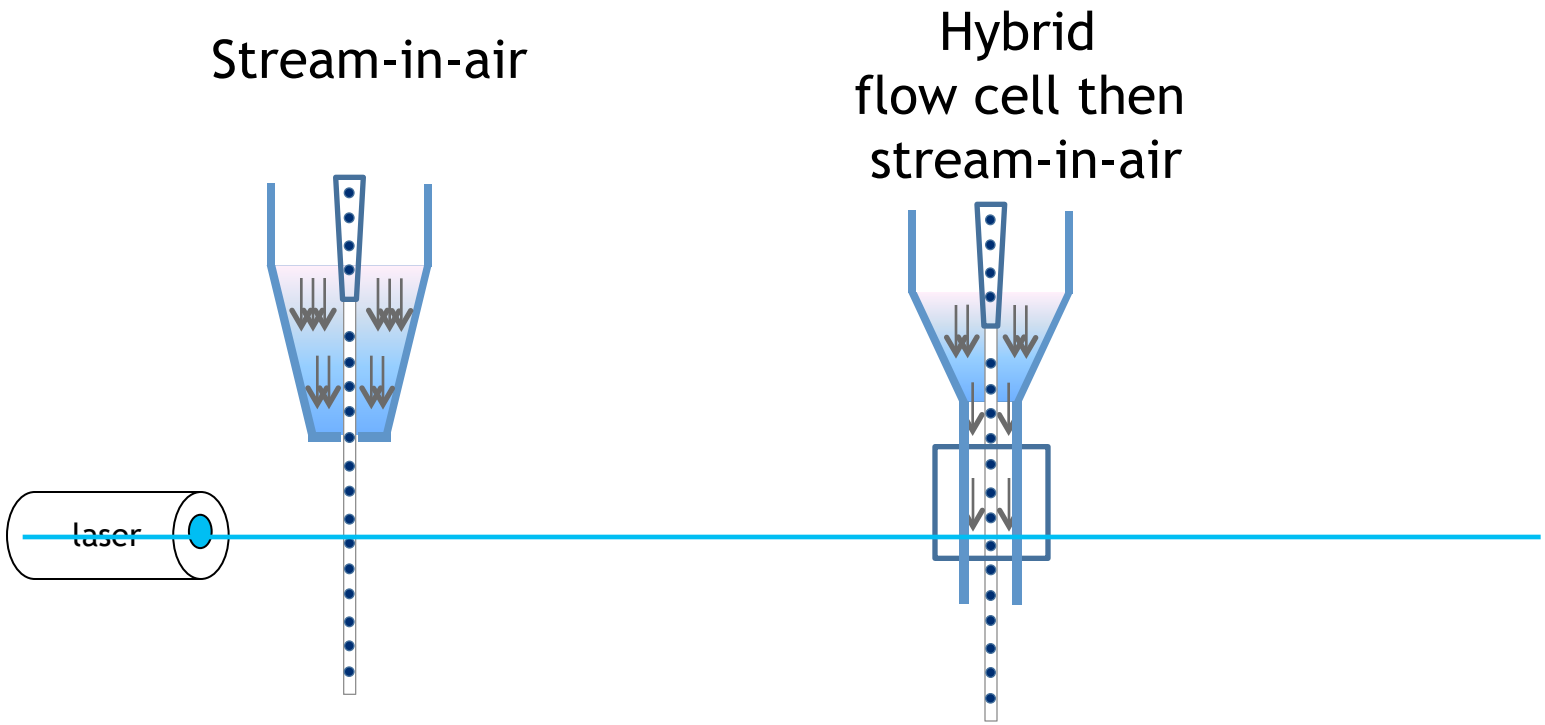
Why would we want to sort cells?

We have a very mixed population of cells



And we want to do experiments with a pure subset of these DC cells

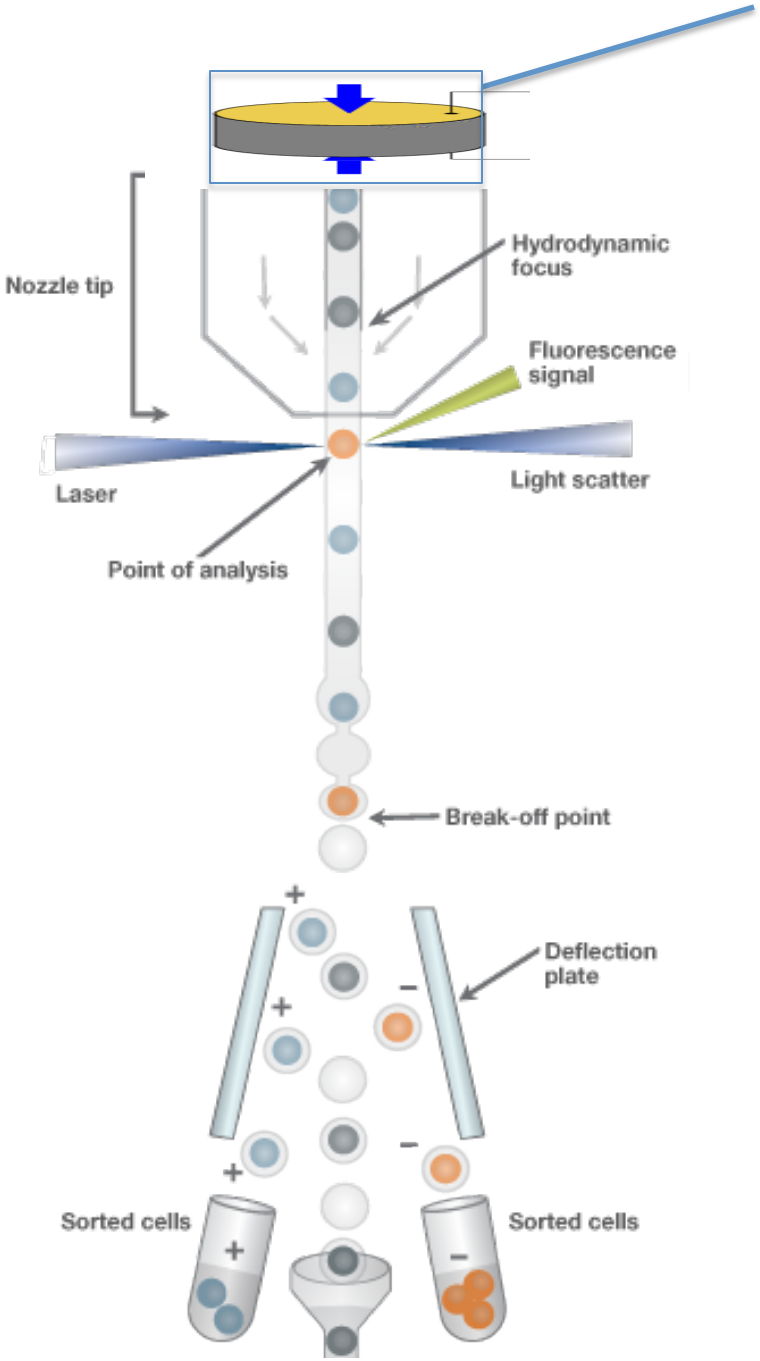
Most sorters are “stream in air”



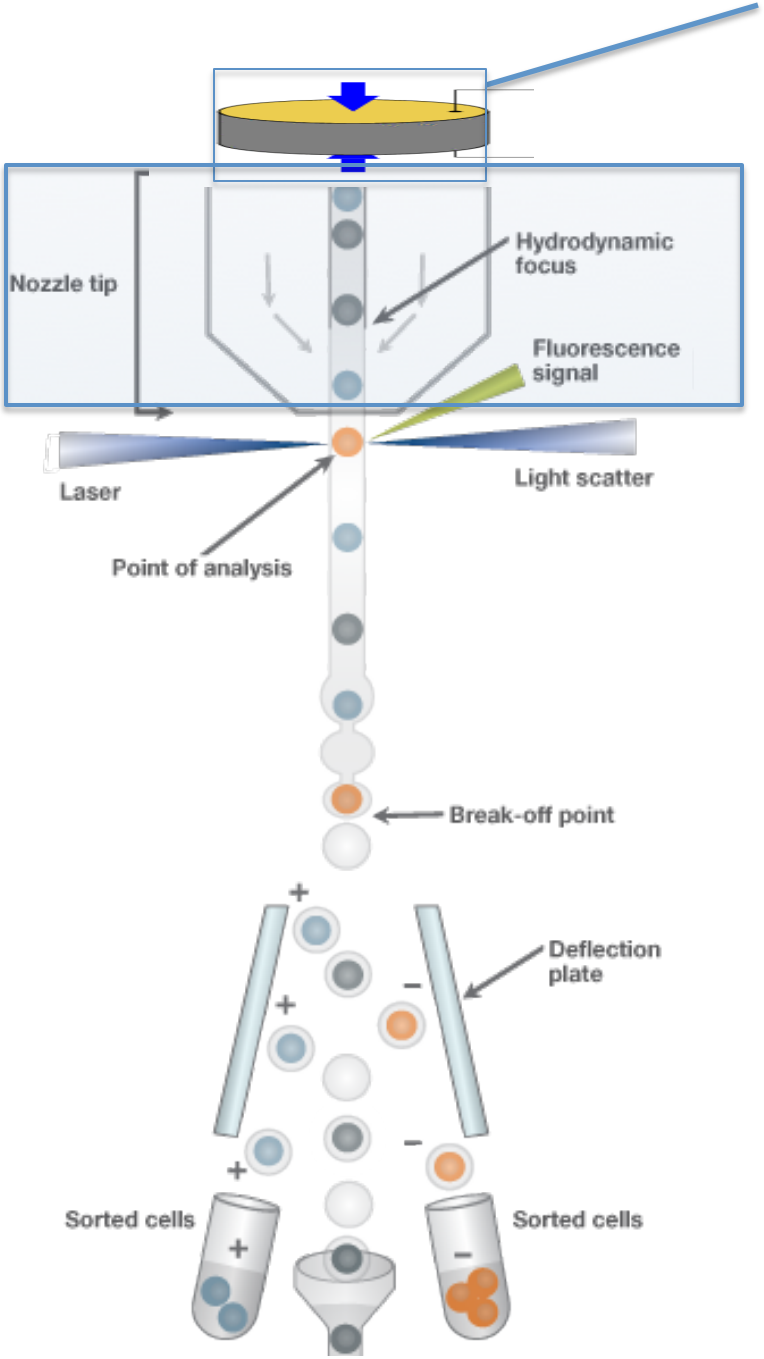
Astrios
S3
Moflo

Aria
Melody

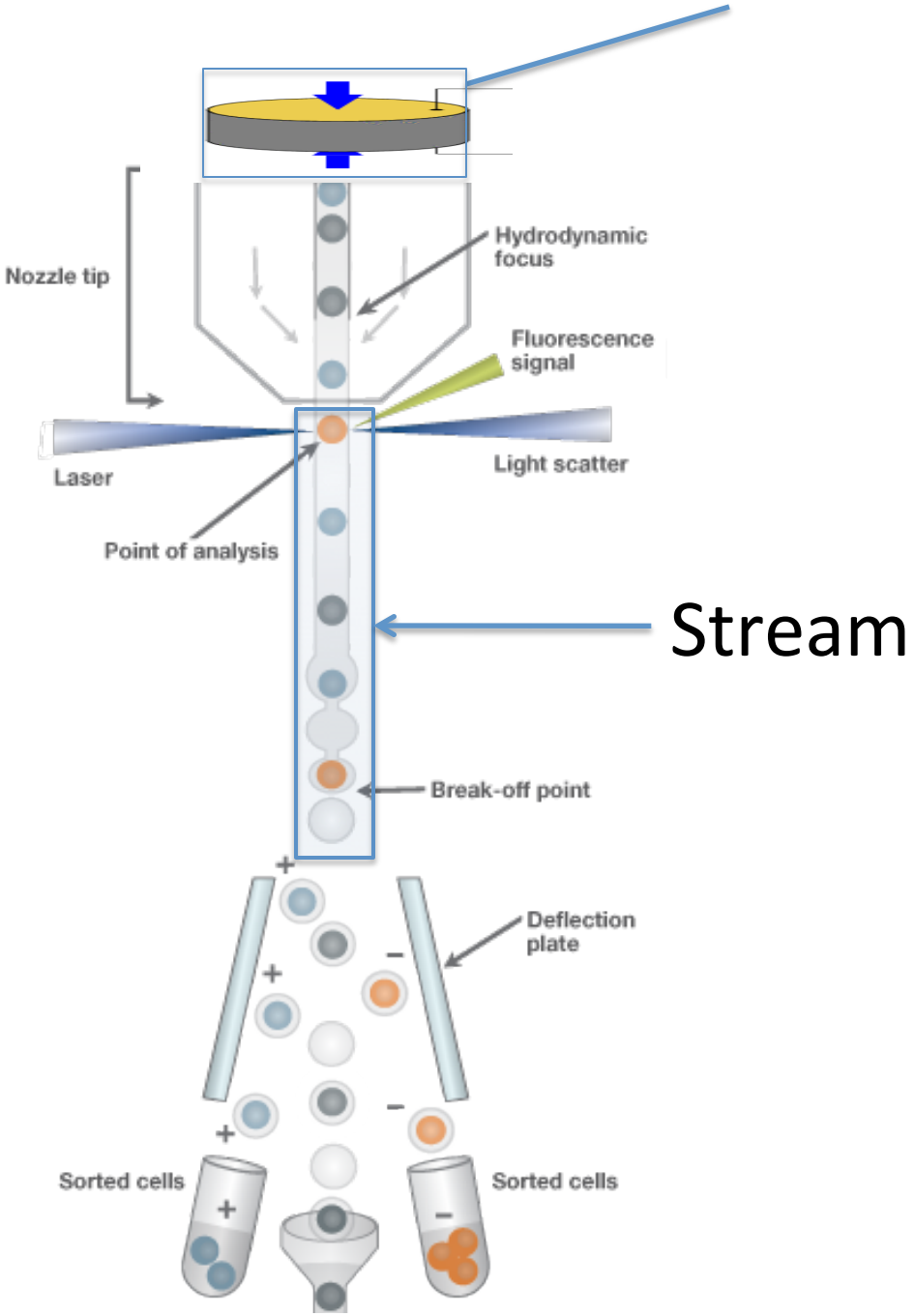
Slide from Bill Telford NIH

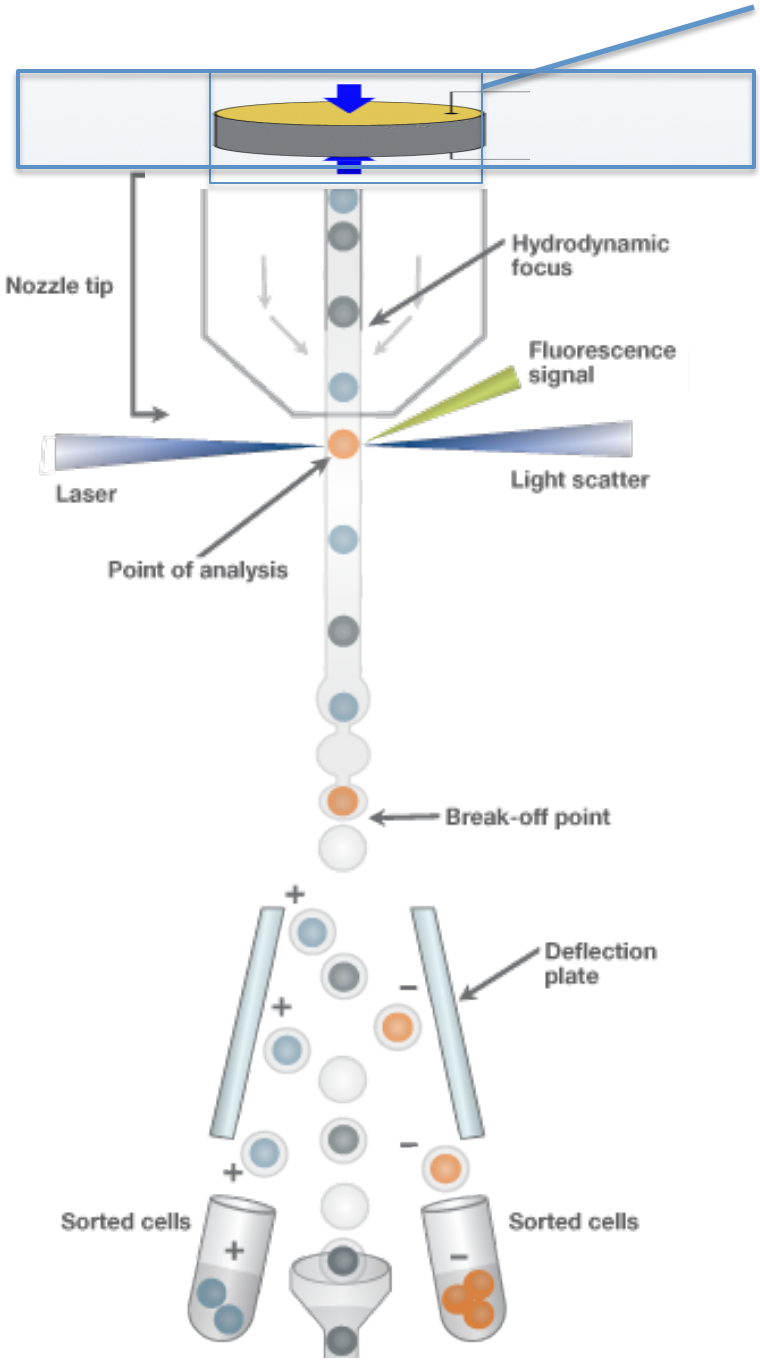


Elements of a Sorter


Nozzle tip
Hydrodynamic focus
Fluorescence signal
Laser
Light scatter
Point of analysis
Break-off point
Deflection plate
Sorted cells
Sorted cells

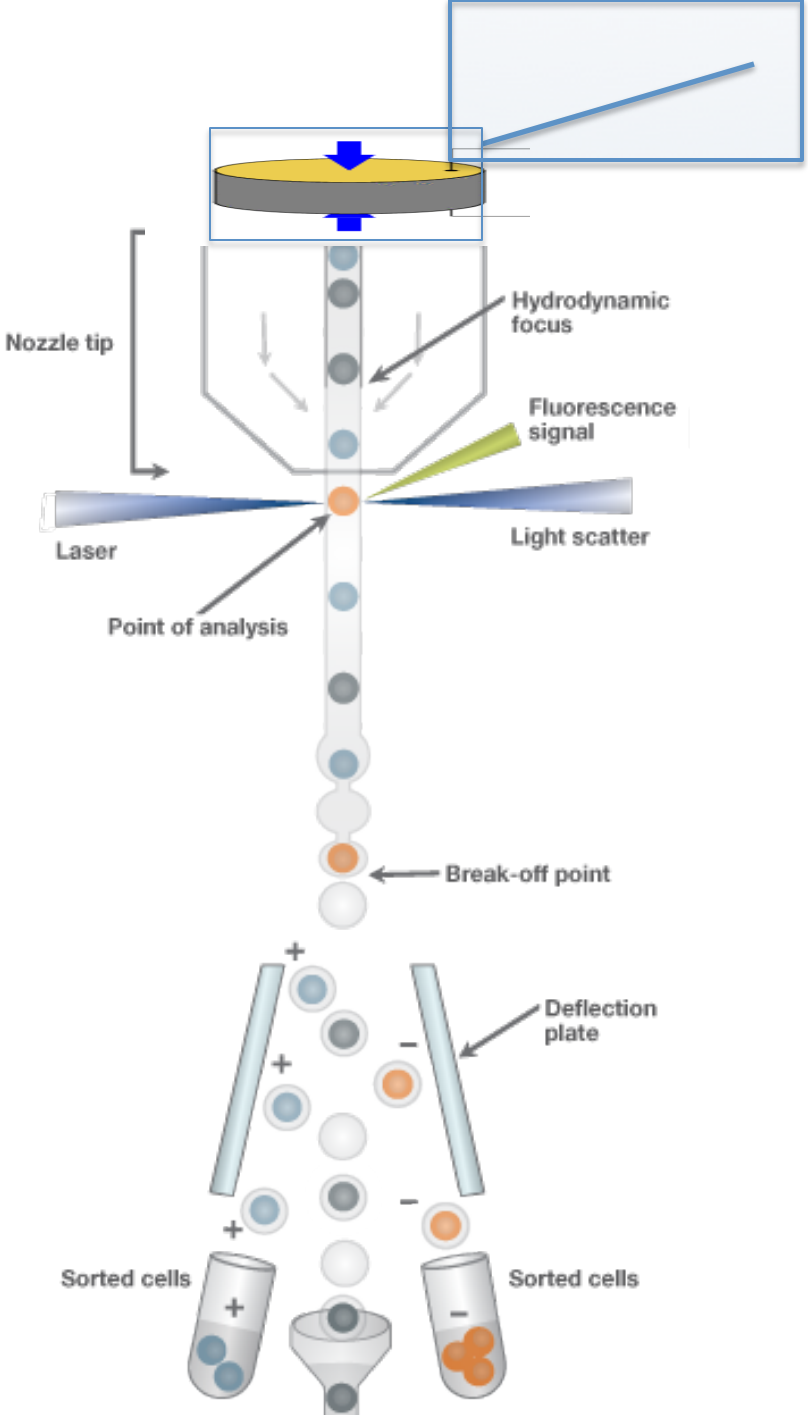
Nozzle

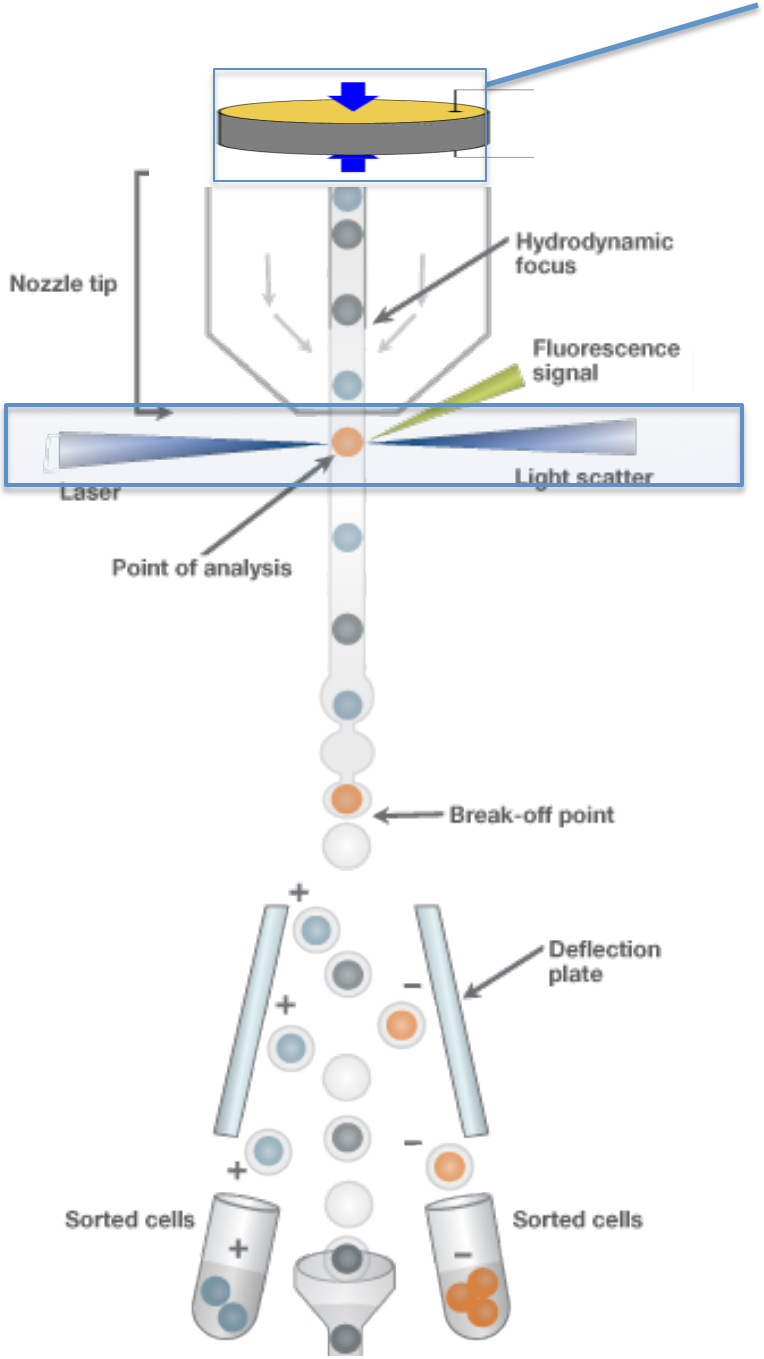

Nozzle tip
Hydrodynamic focus
Fluorescence signal
Laser
Light scatter
Point of analysis
Break-off point
Deflection plate
Sorted cells
Sorted cells
Stream



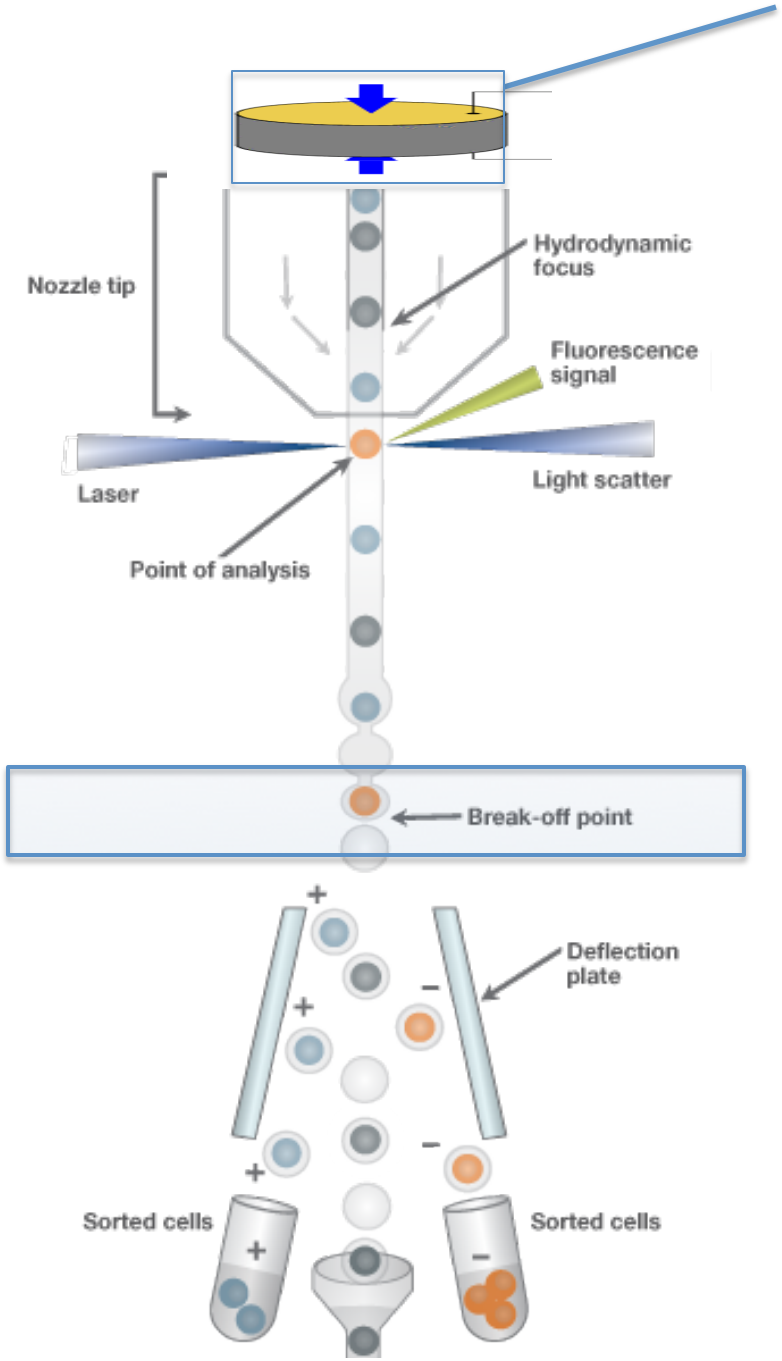
Piezoelectric crystal

Charging wire

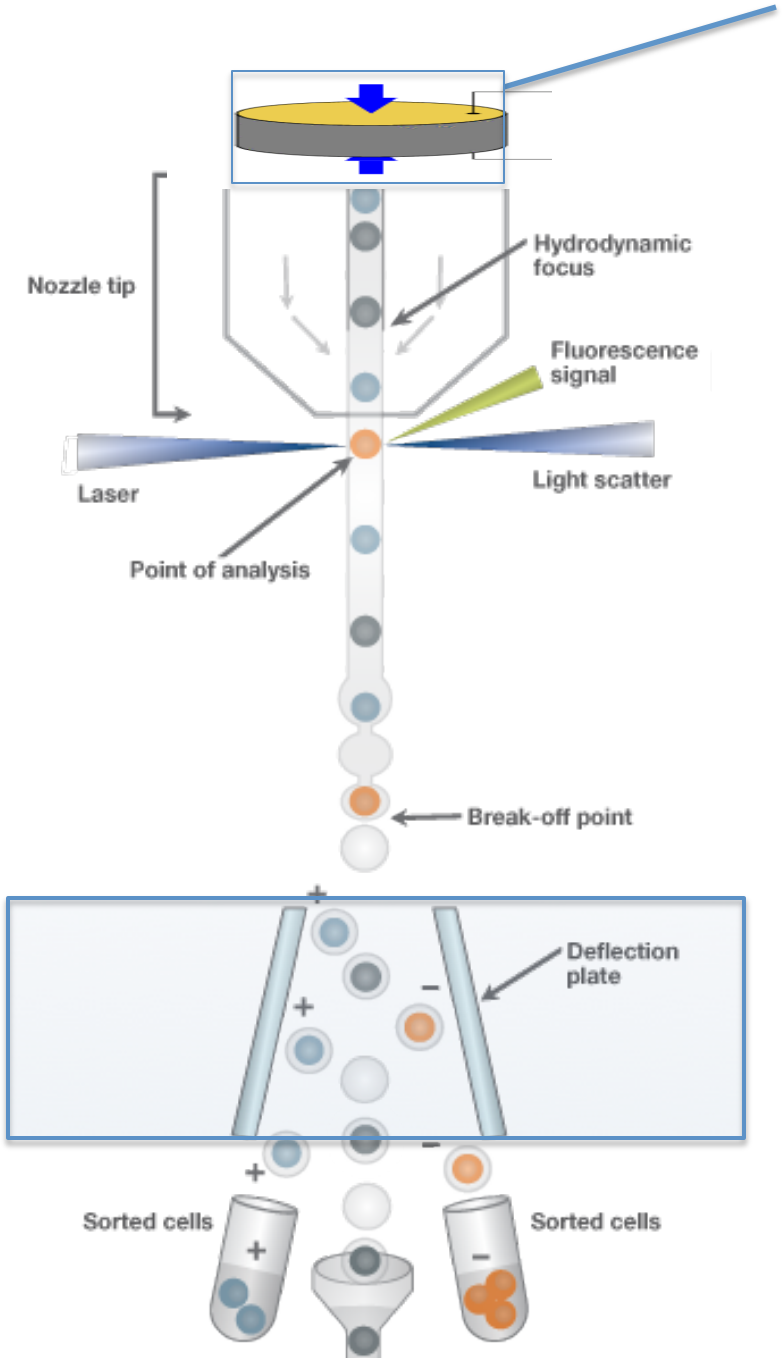




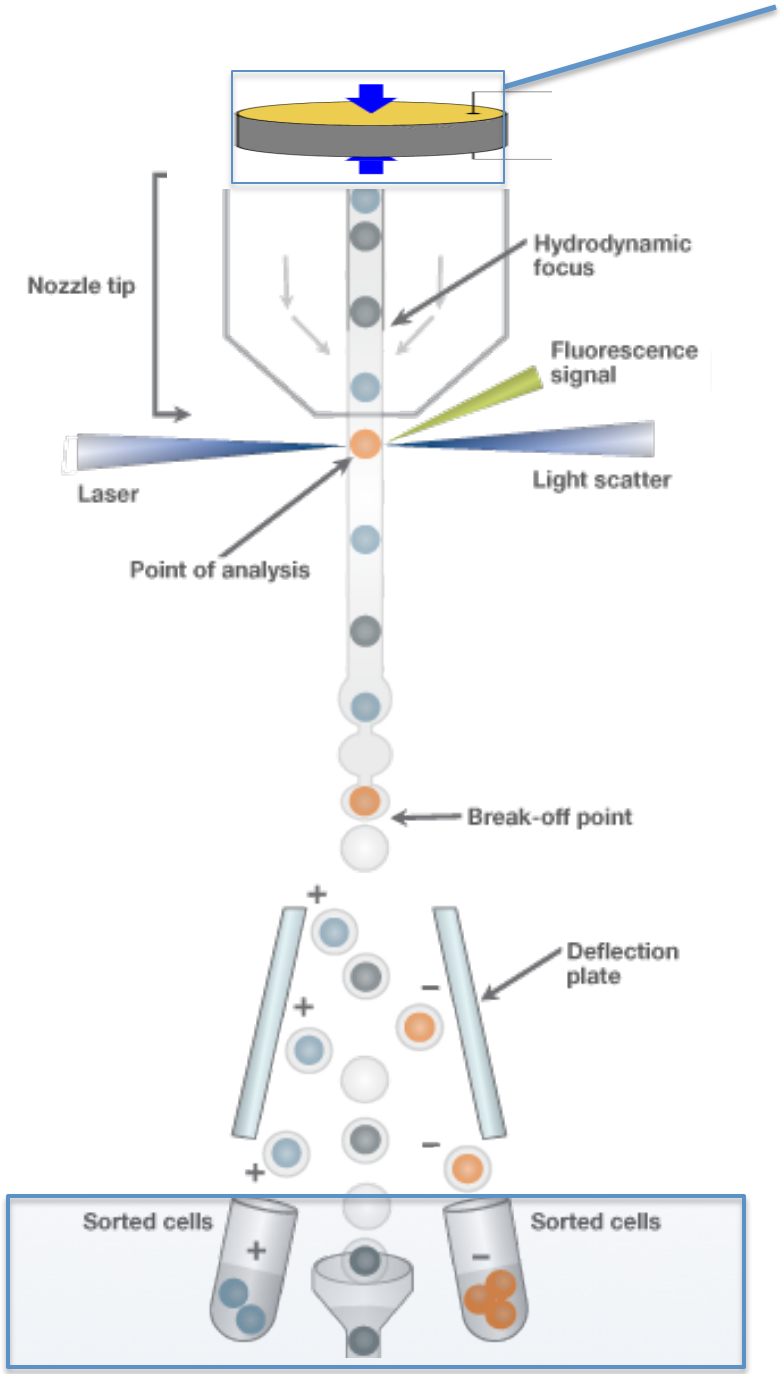
Laser Intercept



Droplet formation and Breakoff Point

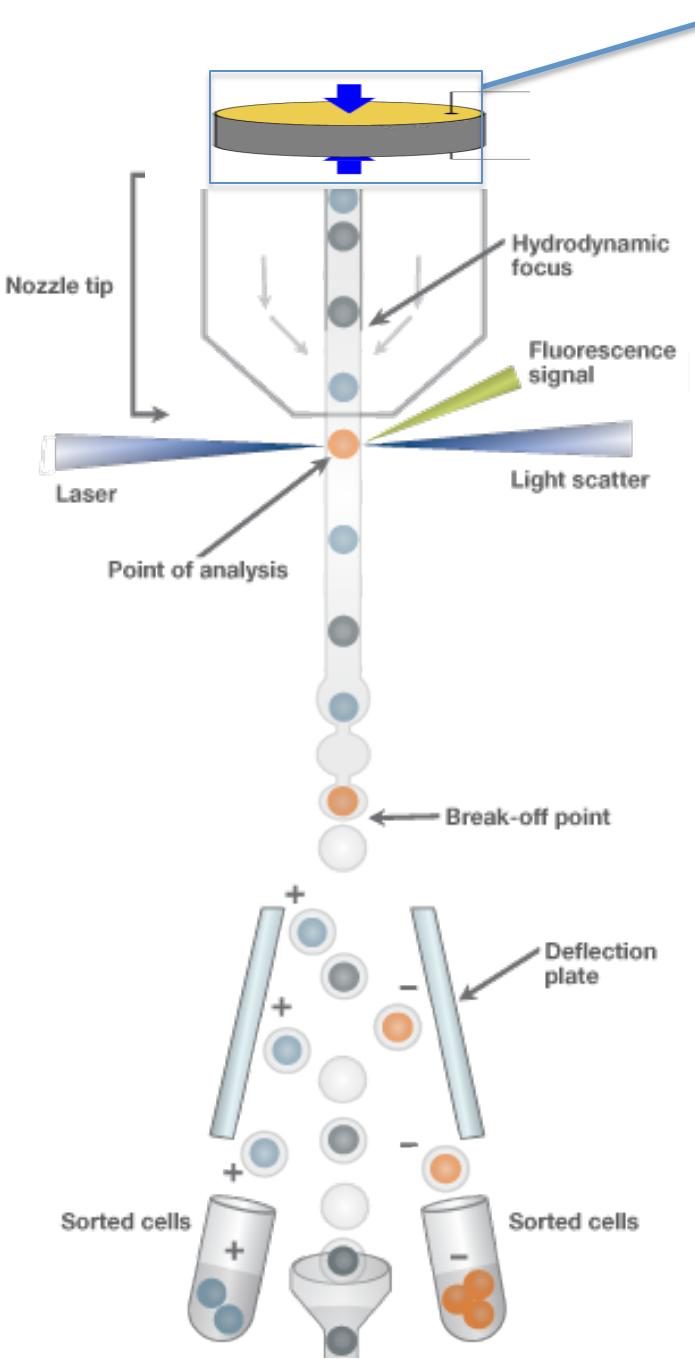


Deflection Plates



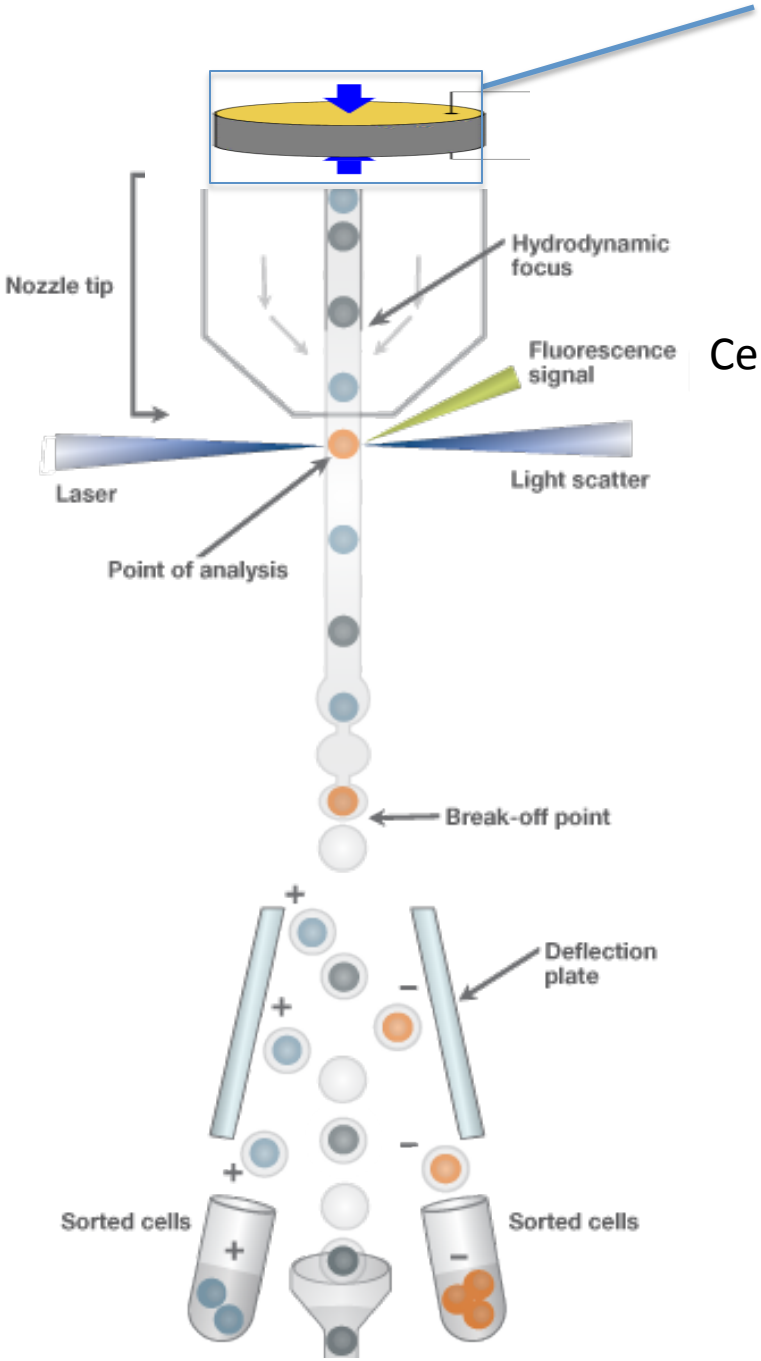
Collection Tubes

How does it work?



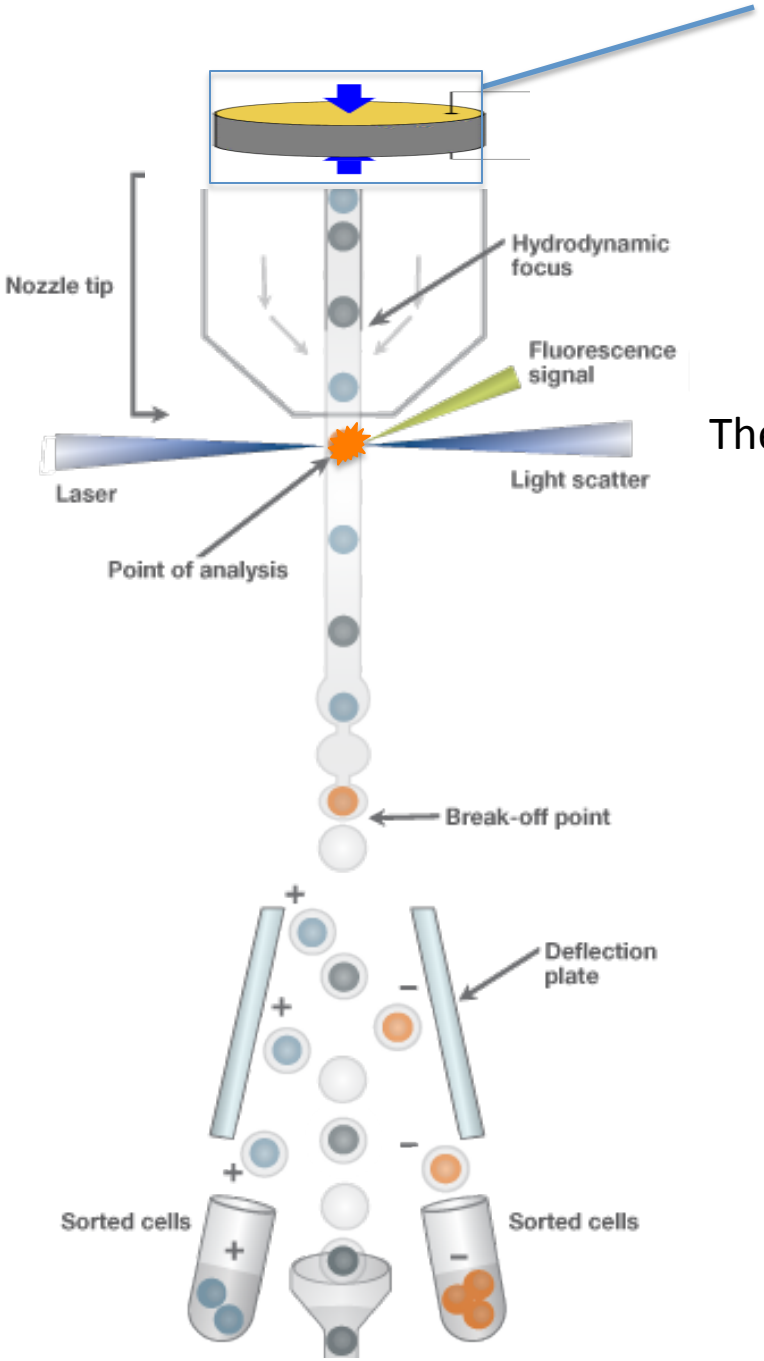
Fluid is pushed out the nozzle tip by pressure to form a stream

An oscillation is applied by the piezoeletric crystal to make waves in the stream so that it breaks into droplets

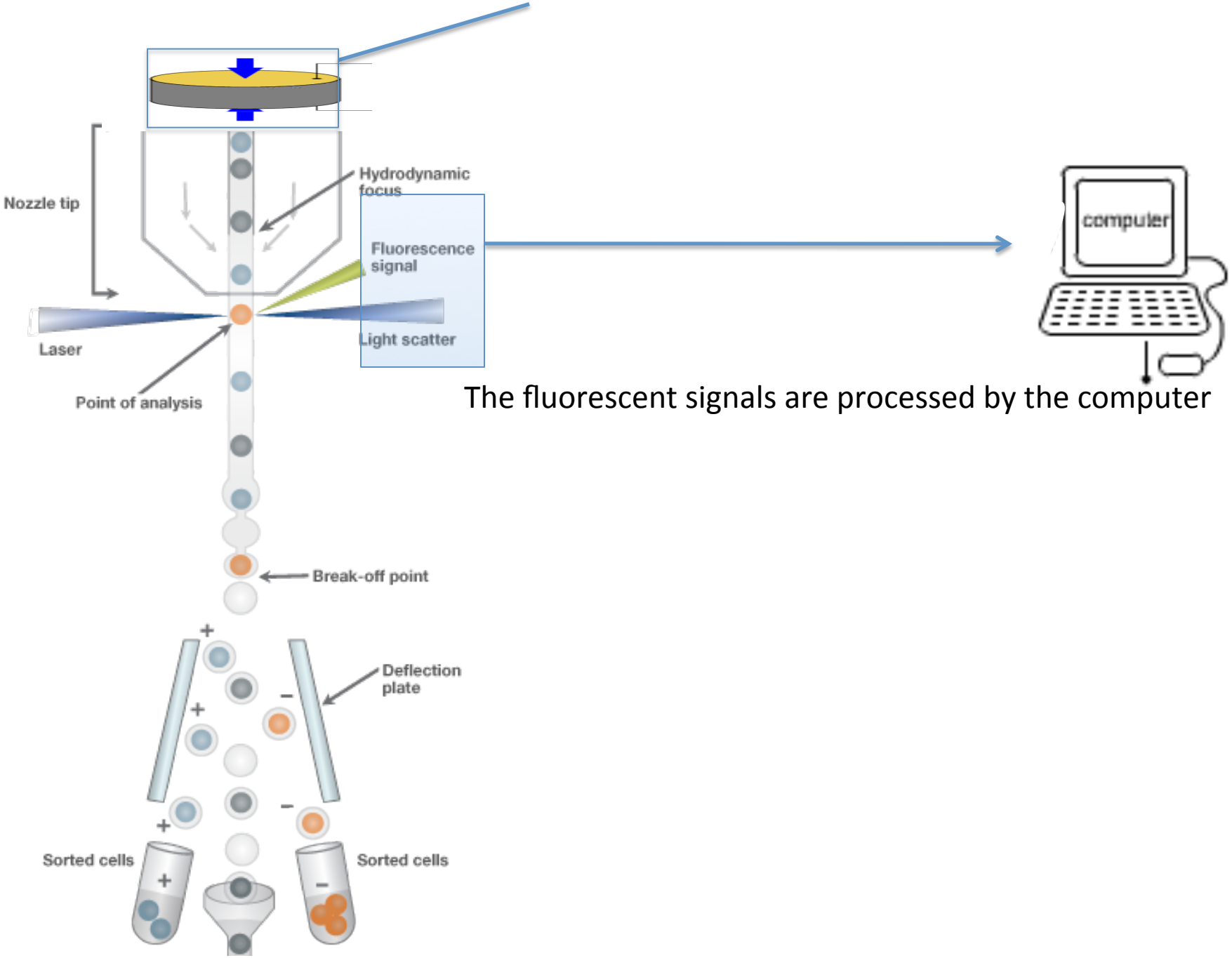


Cells pass one by one through the nozzle into the stream

Detection

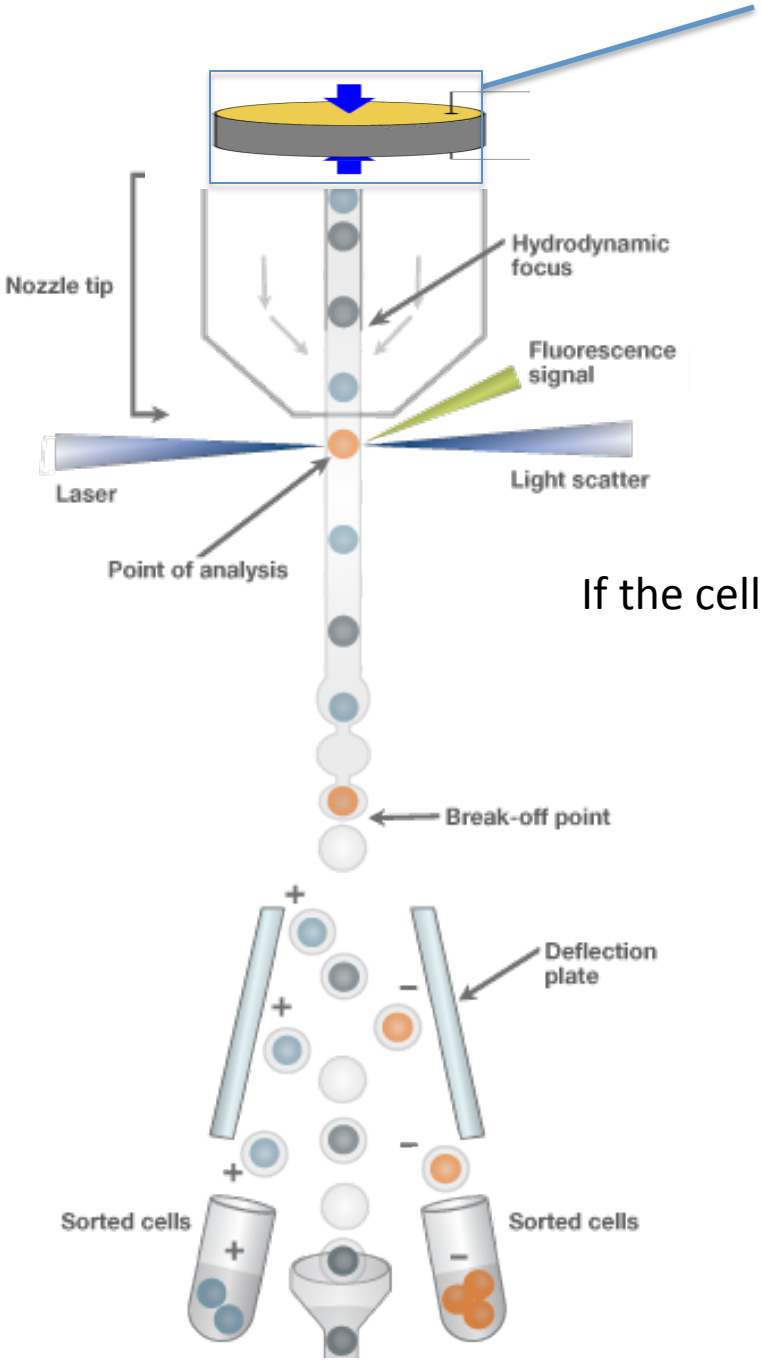


The cells pass through the laser beam and fluoresce

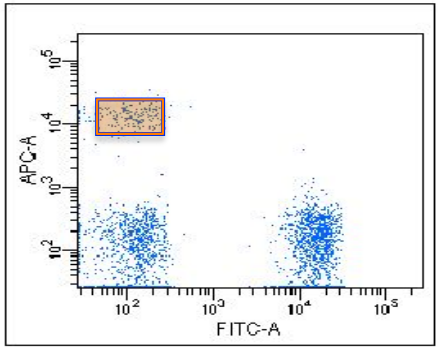


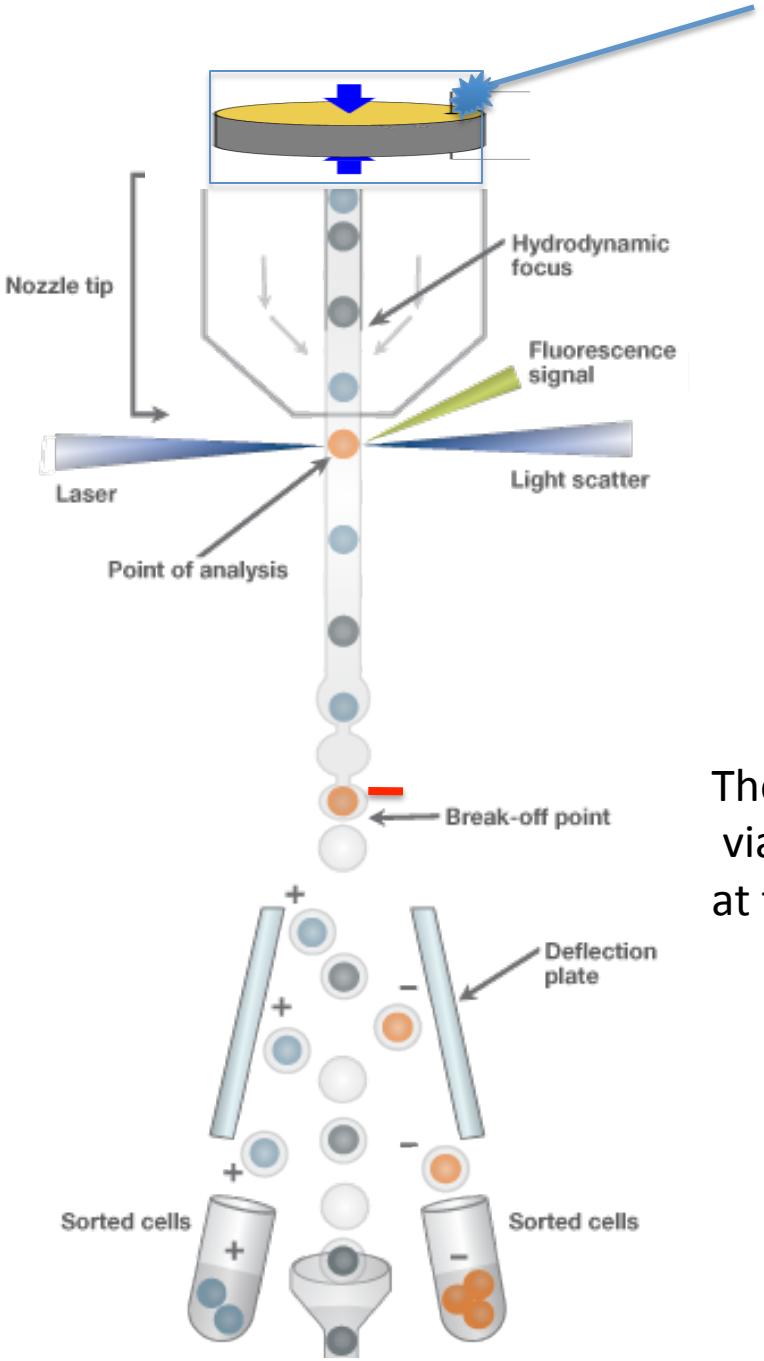
The fluorescent signals are processed by the computer

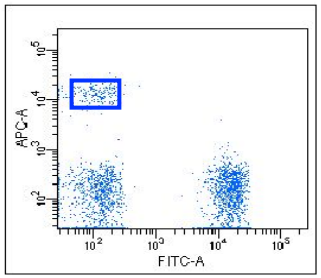
Decision



If the cell is within the defined sort gate

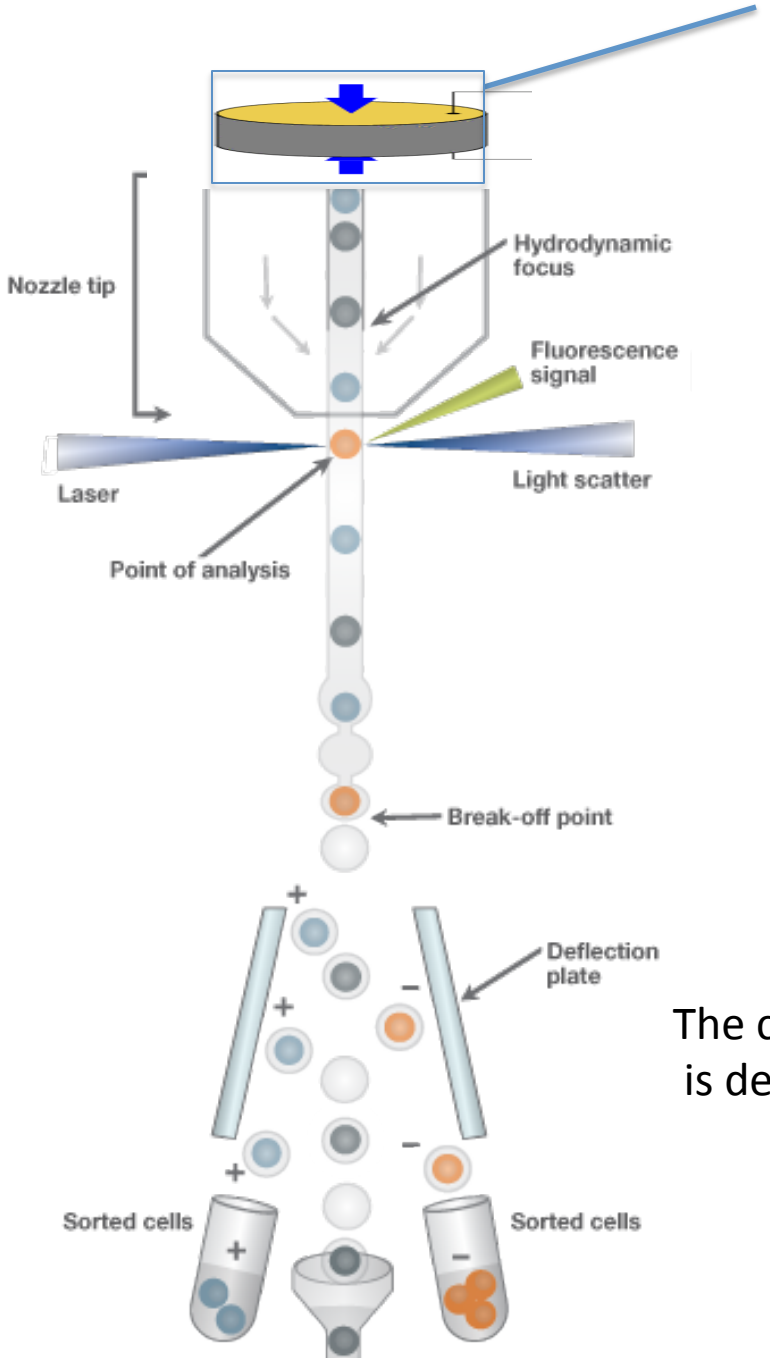






The cytometer sends a signal to charge the stream
via a charging wire in the nozzle
at the very moment that cell reaches the breakoff point

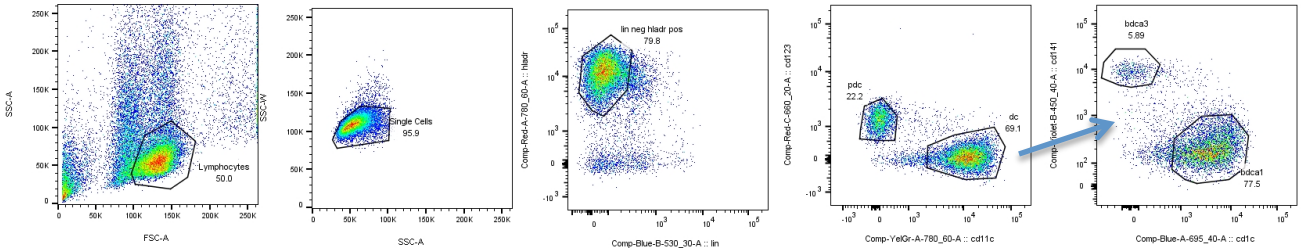
Deflection



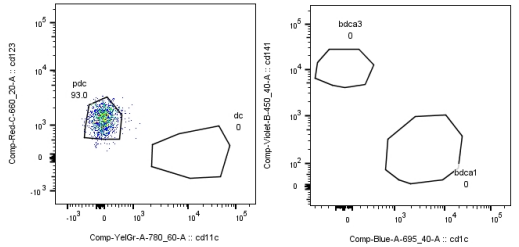
The charged droplet containing that cell
is deflected by charged plates into a collection tube

Sort results

Before sort

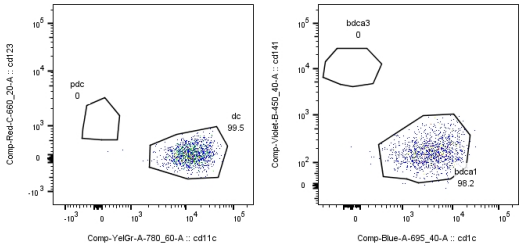


after sort purity checks pdcs

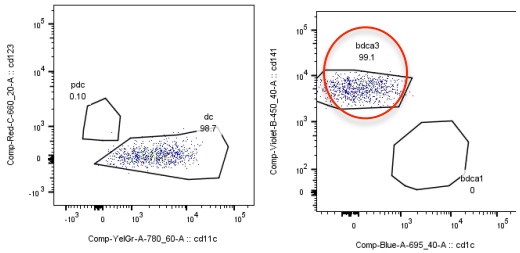


All this happens at
8,000-40,000 cells per second!

dcs bdca1



dcs bdca3



What can Flow Cytometry do?

The cells can be stained with multiple markers coupled to different fluorochromes, up to 28 different colors have been done!

The data acquired allows rapid quantitation and complex analysis of all the different populations of cells in the sample.

Pure subpopulations of cells of interest can sorted at high speed into tubes or or cloned in 96 or 384 well plates for subsequent experimentation.

Applications include multicolor phenotyping, measurement of apoptosis, cell cycle, cell kinetics, minimum residual disease, stem cell analysis, to name but a few.

References

Mike Ormerod's Basic Flow Cytometry book:
http://flowbook.denovosoftware.com/Flow_Book

Howard Shapiro's Flow Cytometry book:
http://www.beckmancoulterreagents.com/us/?page_id=1660

Good basic tutorials free on the web:
https://www.thermofisher.com/fr/fr/home/support/tutorials.html?cid=cid-mptutorials

https://www.bdbiosciences.com/us/support/training/s/itf_launch

Basic data analysis and presentation

Tomáš Kalina

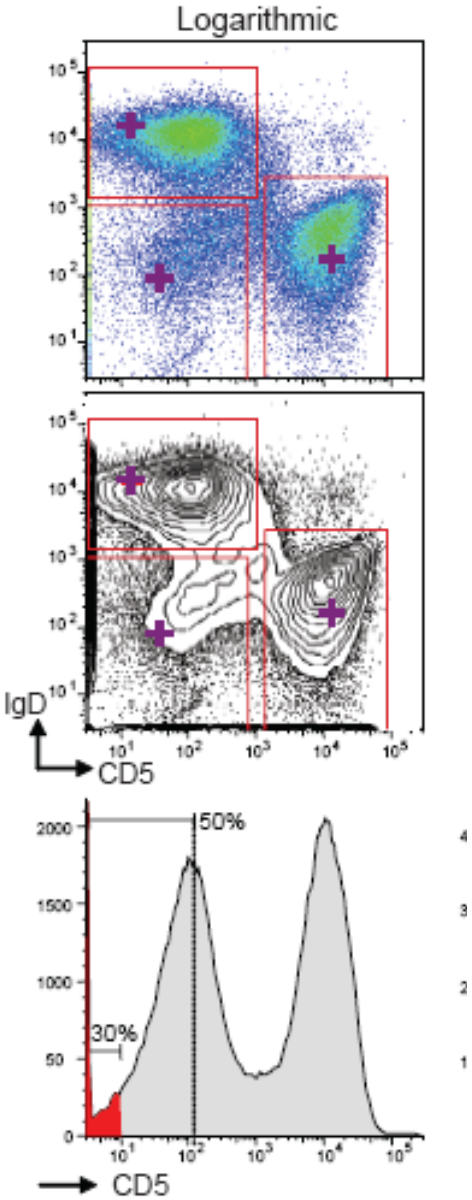
Charles University, 2nd Faculty of Medicine,
Prague, Czech Republic
Dpt. of Pediatric Hematology and Oncology

CLIP - *Childhood Leukemia Investigation Prague*

Outline

- Graphs and visualisation
- Gating controls
- FCS datafiles – keywords
- Complex data visualisation (data reduction: PCA, tSNE)

Data display - graphs

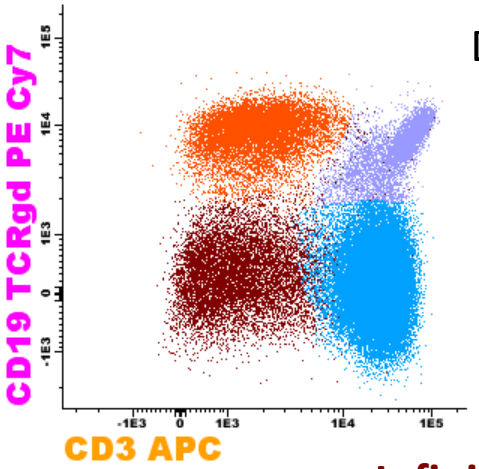


Pseudocolor plot

Contour plot

Histogram

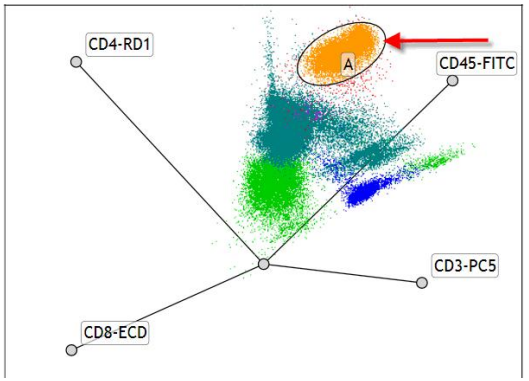
Flowjo software (Mac)



Dot plot

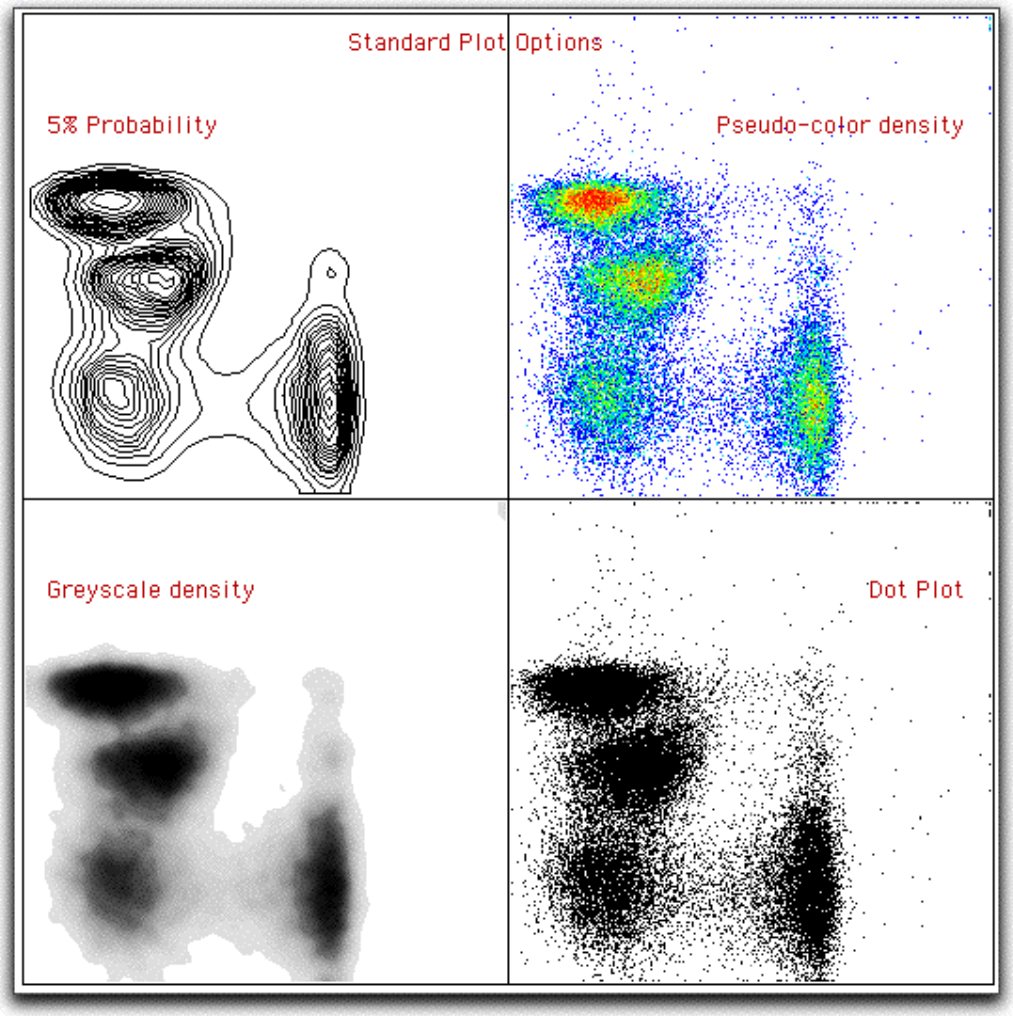
Infinicyt software

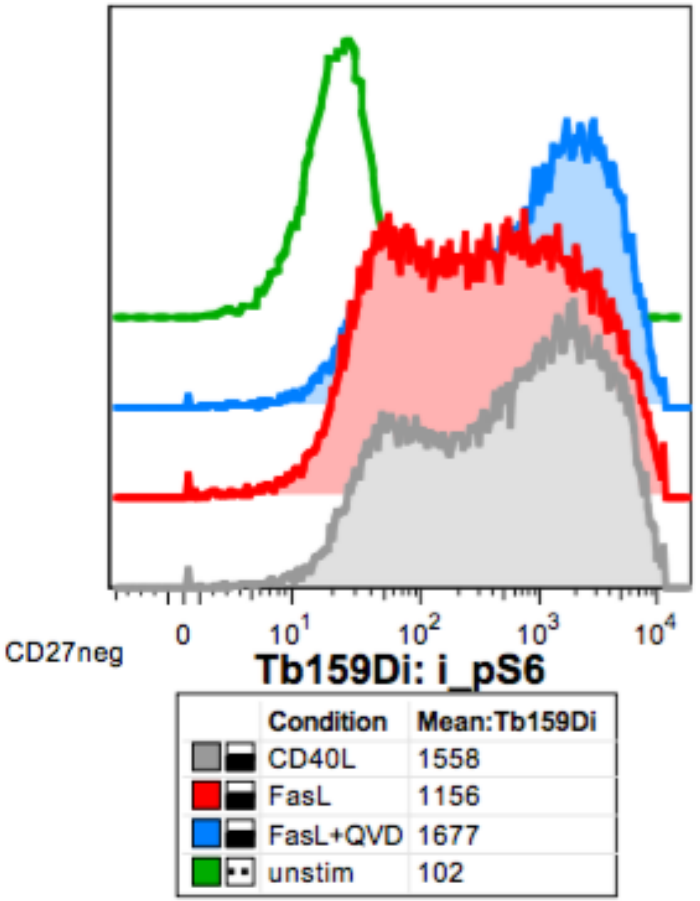
Radar Plot



Kaluza

Basic graphs





FlowJo (Mac)

Data display - logicle

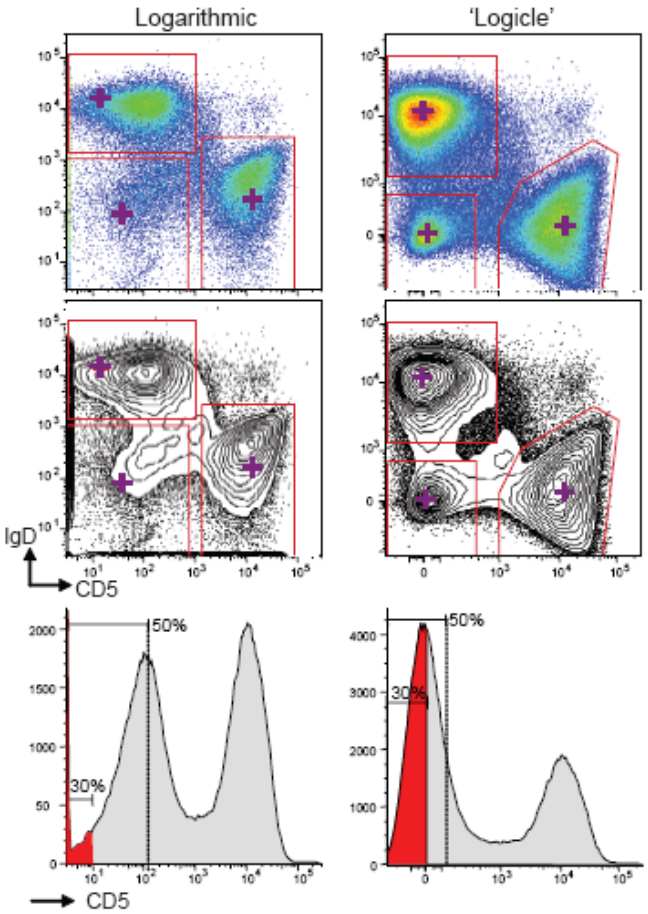


Figure 1 'Logicle' displays provide improved representation of cells with minimal fluorescence. Cells with minimal fluorescence can be visualized with 'logicle' displays (right) but are 'piled up' on the axis with logarithmic displays (left). The true center of each gated population (median fluorescence value in each dimension; dark red crosses) matches the visual peak for that population in 'logicle' displays (right, top and middle) but does not match the visual peak in the logarithmic displays (left, top and middle). Because logarithmic scales cannot display cells with zero or negative values, these cells are 'piled up' on the axis in the logarithmic displays. However, they are properly visualized in the 'logicle' display (bottom right, red shaded region). Data provided by E. Ghosn (Stanford University, Stanford, California).

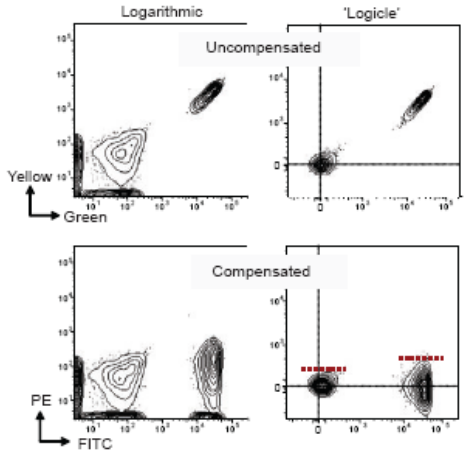
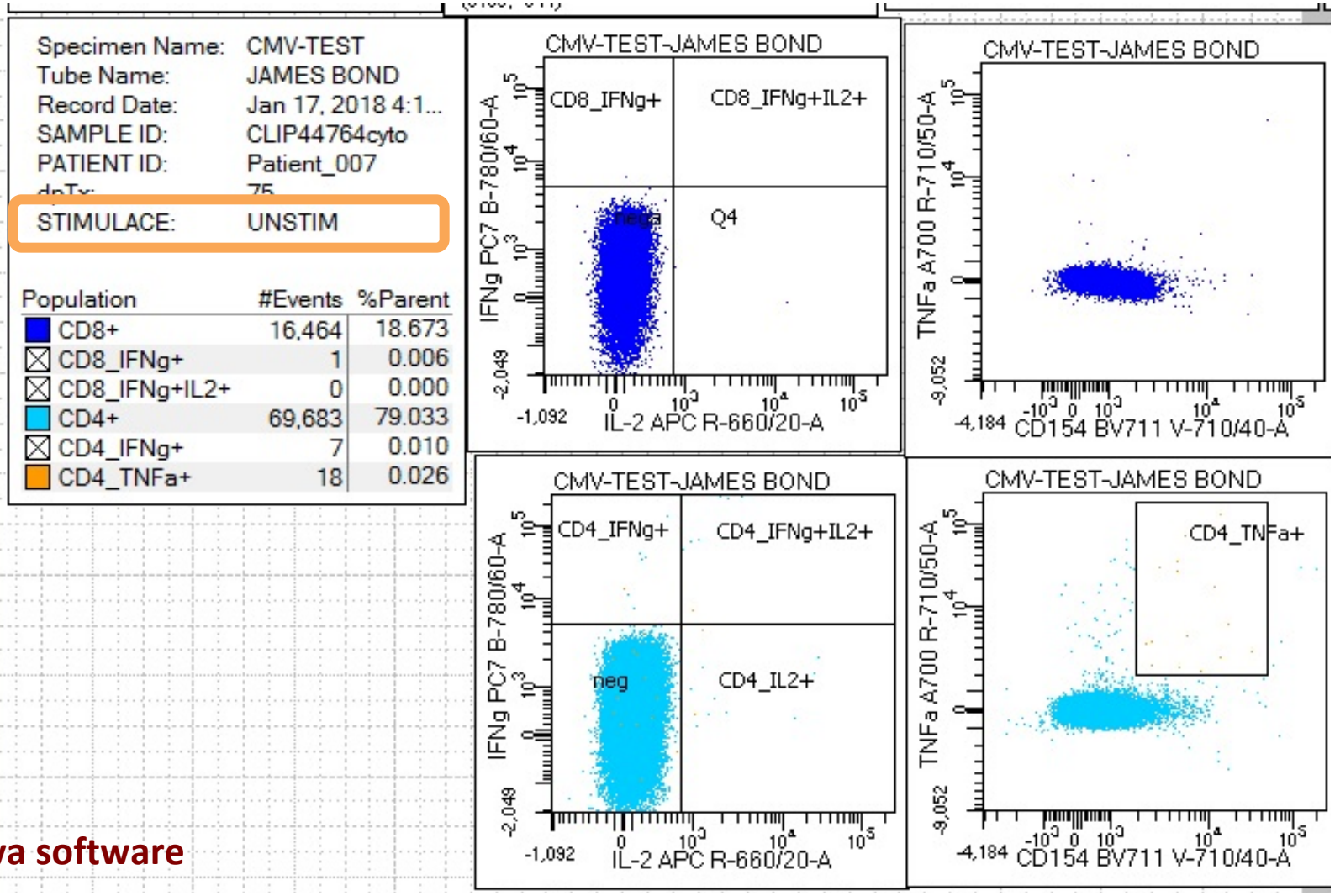


Figure 2 'Logicle' displays are superior to logarithmic displays in determining whether compensation has been properly applied to the sample. Uncompensated data for a sample stained with fluorescein isothiocyanate–conjugated antibody are presented in logarithmic (top left) and 'logicle' (top right) displays. Fluorescent colors (yellow and green) are used to designate the axes because they represent uncompensated color measurements. Many events are 'piled up' on the axis in the logarithmic display, whereas all events are visible in the 'logicle' display. Bottom, compensated data for samples above. In a properly compensated sample, the location of the median phycoerythrin fluorescence in the FITC⁻ population by definition matches the location of the median phycoerythrin fluorescence in the FITC⁺ population. This match is apparent in the 'logicle' display but not the logarithmic display (bottom right versus bottom left). In the 'logicle' display (bottom right), the distribution of the 'phycoerythrin' fluorescence in the FITC⁺ population is broader than the distribution of the low fluorescence measured for the FITC⁻ population. This difference is due to statistical variance of the fluorescence measurements, which increases with the amount of overlap fluorescence that must be subtracted. Red dashed lines above the populations (bottom right) indicate the thresholds (gates) needed to identify the PE⁺ events in the FITC⁺ and FITC⁻ populations in this simple analysis. With more complex stain sets, FMO controls are useful for setting these thresholds.

Other names:
Log-linear transformation
Logicle display
Biexponential (DiVa)
VisiComp (Summit 4,3)

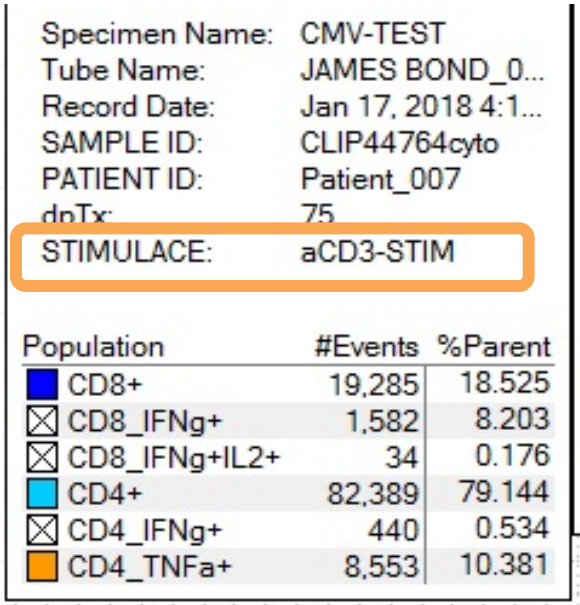
Herzenberg, L. A., J. Tung, et al. (2006). "Interpreting flow cytometry data: a guide for the perplexed." Nat Immunol **7**(7): 681-5.

Gating controls



Diva software

Gating controls



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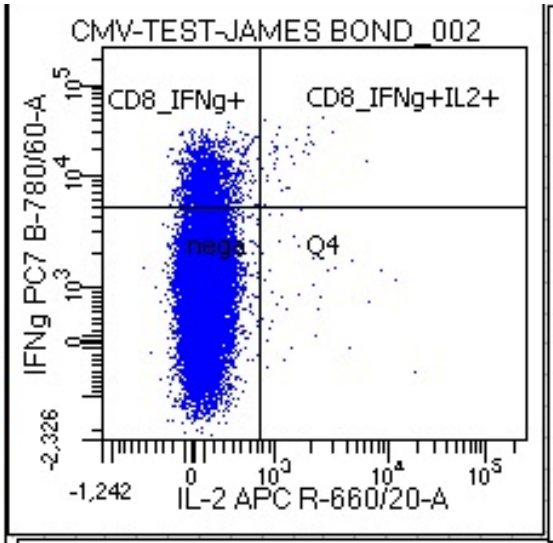
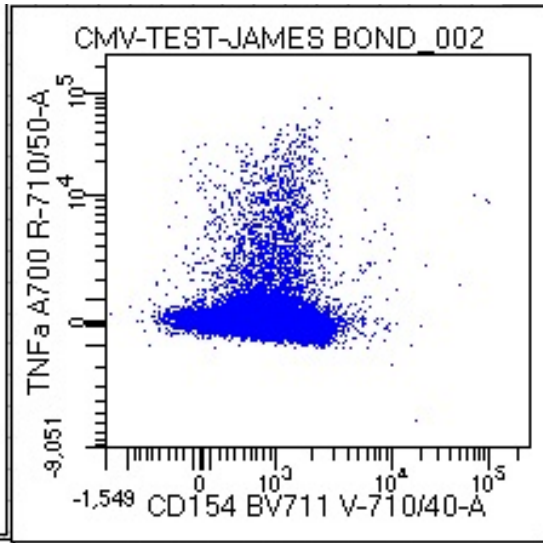
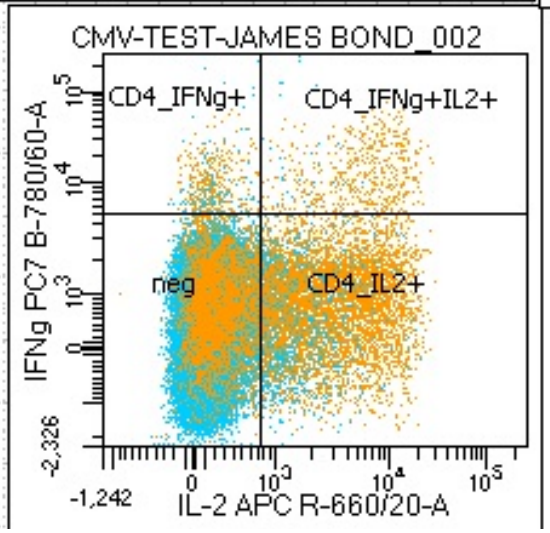
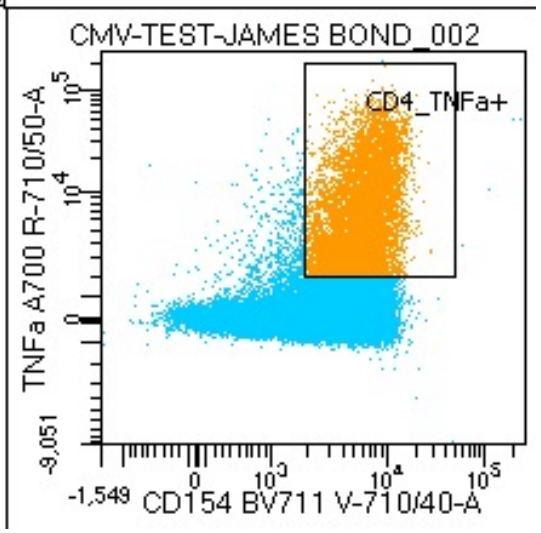
Record Date:	Jan 17, 2018 4:1...
SAMPLE ID:	CLIP44764cyto
PATIENT ID:	Patient_007
dpTx:	75
STIMULACE:	aCD3-STIM

Population	#Events	%Parent
CD8+	19,285	18.525
CD8_IFNg+	1,582	8.203
CD8_IFNg+IL2+	34	0.176
CD4+	82,389	79.144
CD4_IFNg+	440	0.534
CD4_TNFa+	8,553	10.381

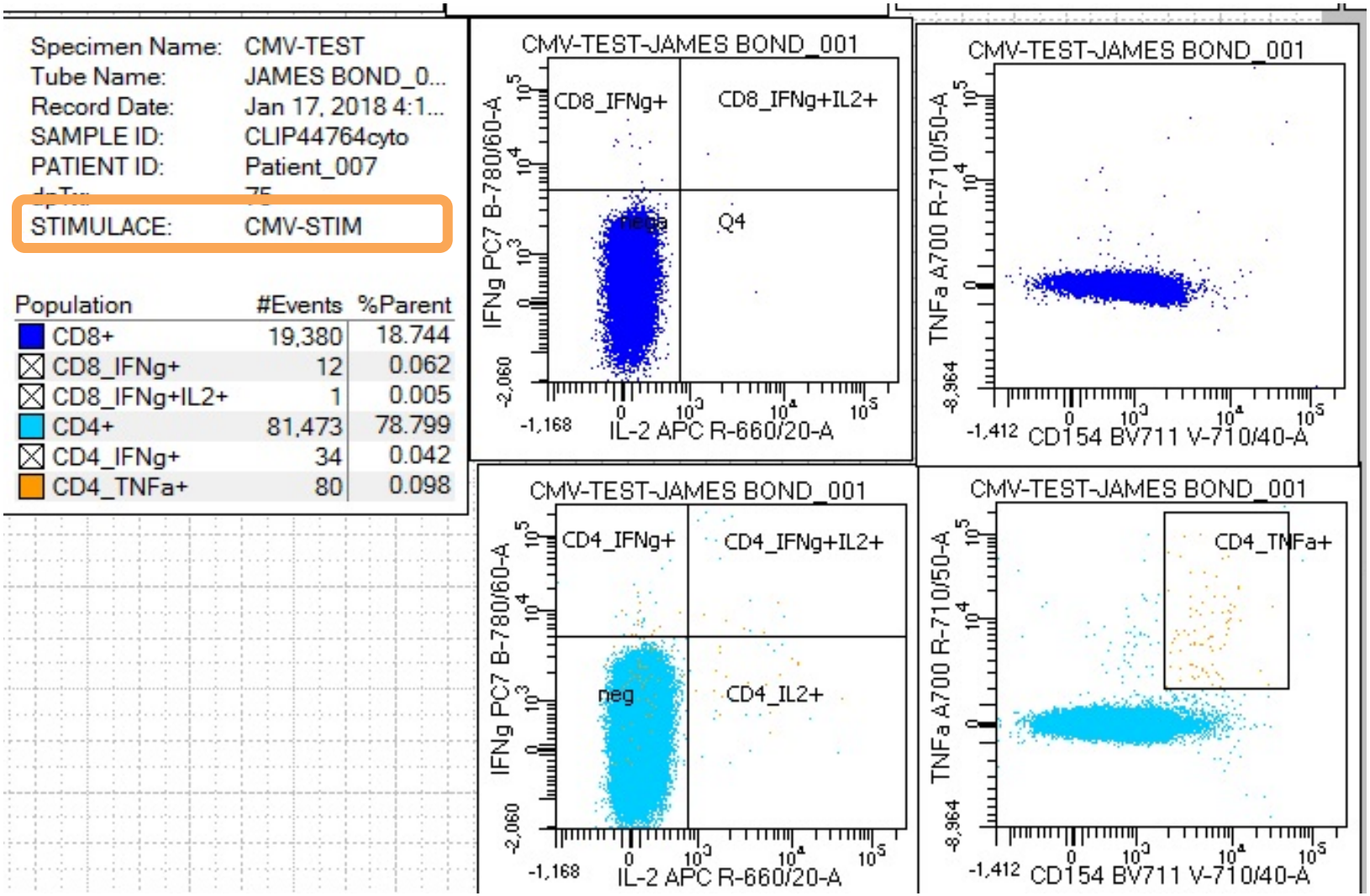








Gating controls



Flowjo software (Mac)

Gating templates

template

Basic concepts

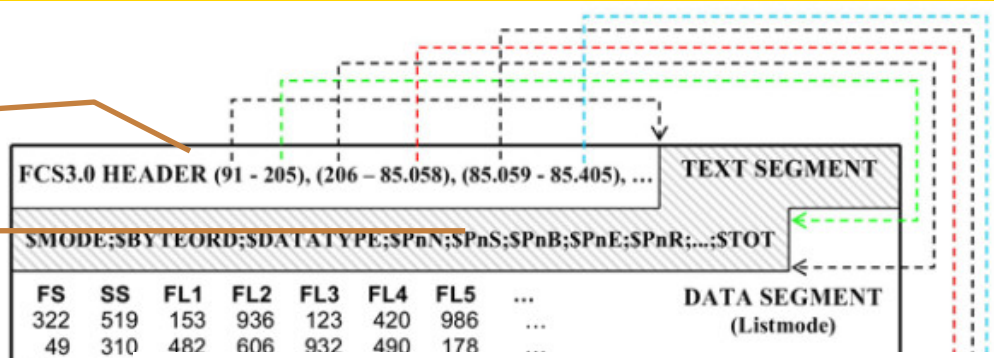
- FCS datafiles
  - What are they, how to use Keywords

What is a FSC datafile ?

HEADER (FSC 3.0) etc (text)

KEYWORDS ($DATE, $TOT, $SAMPLEID….. $P1S, $P1N, $P1V,)

DATA row = cell Column = parameter (compressed)



ORIGINAL ARTICLE

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

Data File Standard for Flow Cytometry, Version FCS 3.1

Josef Spidlen,[1] Wayne Moore,[2] David Parks,[3] Michael Goldberg,[4] Chris Bray,[5] Pierre Bierre,[6] Peter Gorombey,[7] Bill Hyun,[8] Mark Hubbard,[9] Simon Lange,[10] Ray Lefebvre,[11] Robert Leif,[12] David Novo,[13] Leo Ostruszka,[14] Adam Treister,[15] James Wood,[16] Robert F. Murphy,[17] Mario Roederer,[18] Damir Sudar,[19] Robert Zigon,[20] Ryan R. Brinkman[1]*

[1]Terry Fox Laboratory, BC Cancer Agency, Vancouver, British Columbia, Canada

[2]Department of Genetics, Stanford University School of Medicine, Stanford, California, USA

[3]Stanford Shared FACS Facility, Stanford University, Stanford, California, USA

• **Abstract**

The flow cytometry data file standard provides the specifications needed to completely describe flow cytometry data sets within the confines of the file containing the experimental data. In 1984, the first Flow Cytometry Standard format for data files was adopted as FCS 1.0. This standard was modified in 1990 as FCS 2.0 and again in 1997 as FCS 3.0. We report here on the next generation flow cytometry standard data file format. FCS 3.1 is a minor revision based on suggested improvements from the community. The unchanged goal of the standard is to provide a uniform file format that allows files created by one type of acquisition hardware and software to be analyzed by any other type.

KEYWORDS in FCS

$DATE acquisition date
$BTIM Clock time
$TOT event count

$SAMPLEID
$PATIENT ID
$FIL orig file name

$P1S, $P1N, $P1V,

$SPILLOVER comp matrix

$BTIM Clock time at beginning of data acquisition.
….
$CYT Type of flow cytometer.
$CYTSN Flow cytometer serial number.
$DATE Date of data set acquisition.
$ETIM Clock time at end of data acquisition.

$EXP Name of investigator initiating the experiment.
$FIL Name of the data file containing the data set.

$OP Name of flow cytometry operator

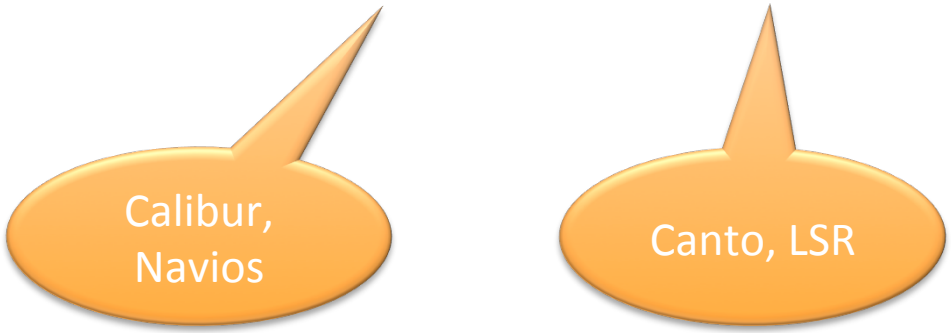
$SPILLOVER Fluorescence spillover matrix.

http://isac-net.org/PDFS/90/9090600d-19be-460d-83fc-f8a8b004e0f9.pdf

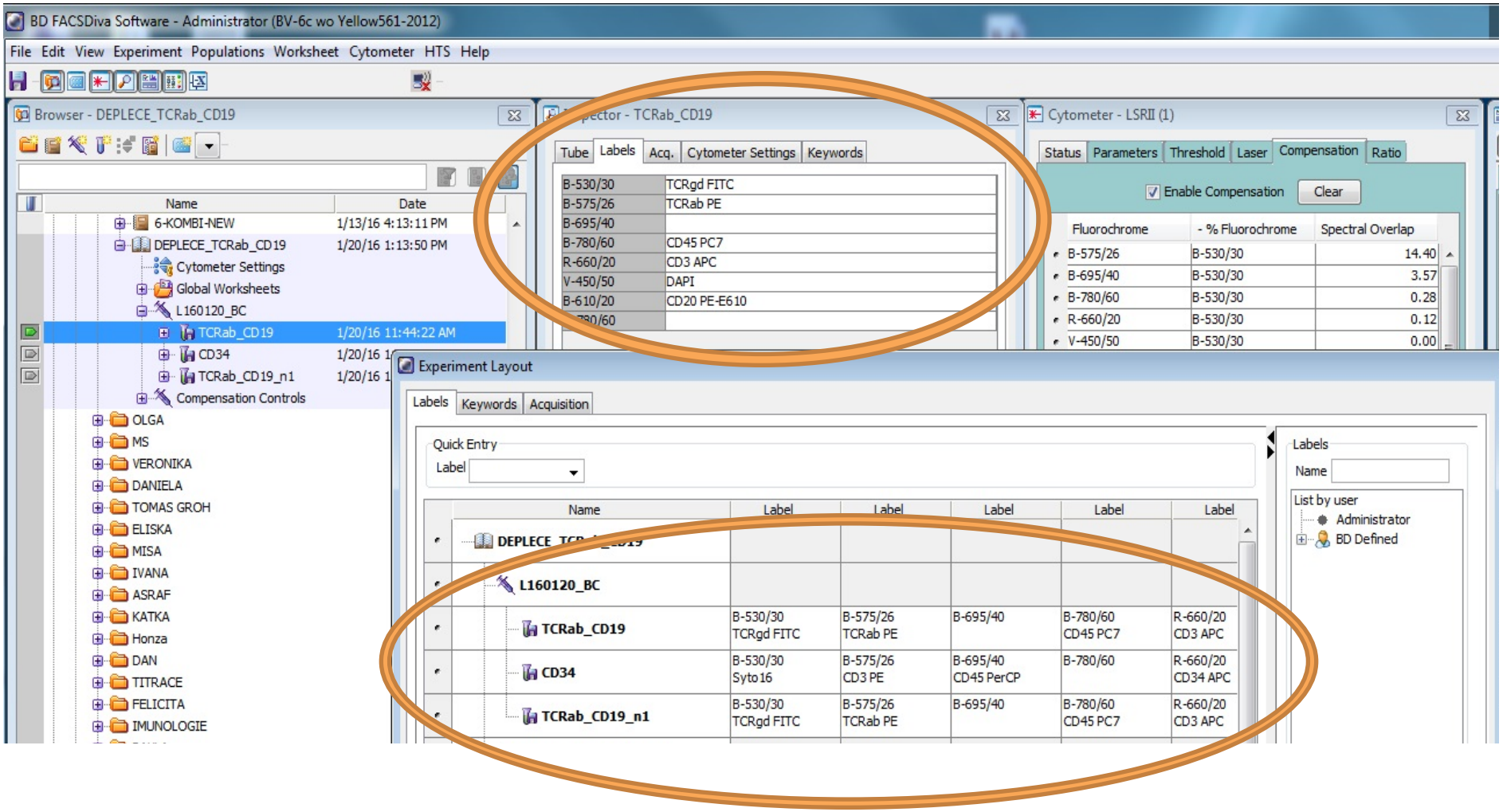
KEYWORDS in FCS

Parameters in FCS (“labels”):

$P1N	… par name….	FL1	FITC	**B-530-30**
$P1S	… par stain ….	CD3 FITC	CD3	**CD3 FITC**
$P1V	. par voltage ..	550	550	**550**



Labels (Diva)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MS
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KATKA
Honza
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TITRACE
FELICITA
IMUNOLOGIE
Tube Labels Acq. Cytometer Settings Keywords
B-530/30 TCRgd FITC
B-575/26 TCRab PE
B-695/40
B-780/60 CD45 PC7
R-660/20 CD3 APC
V-450/50 DAPI
B-610/20 CD20 PE-E610
Cytometer - LSRII (1)
Status Parameters Threshold Laser Compensation Ratio
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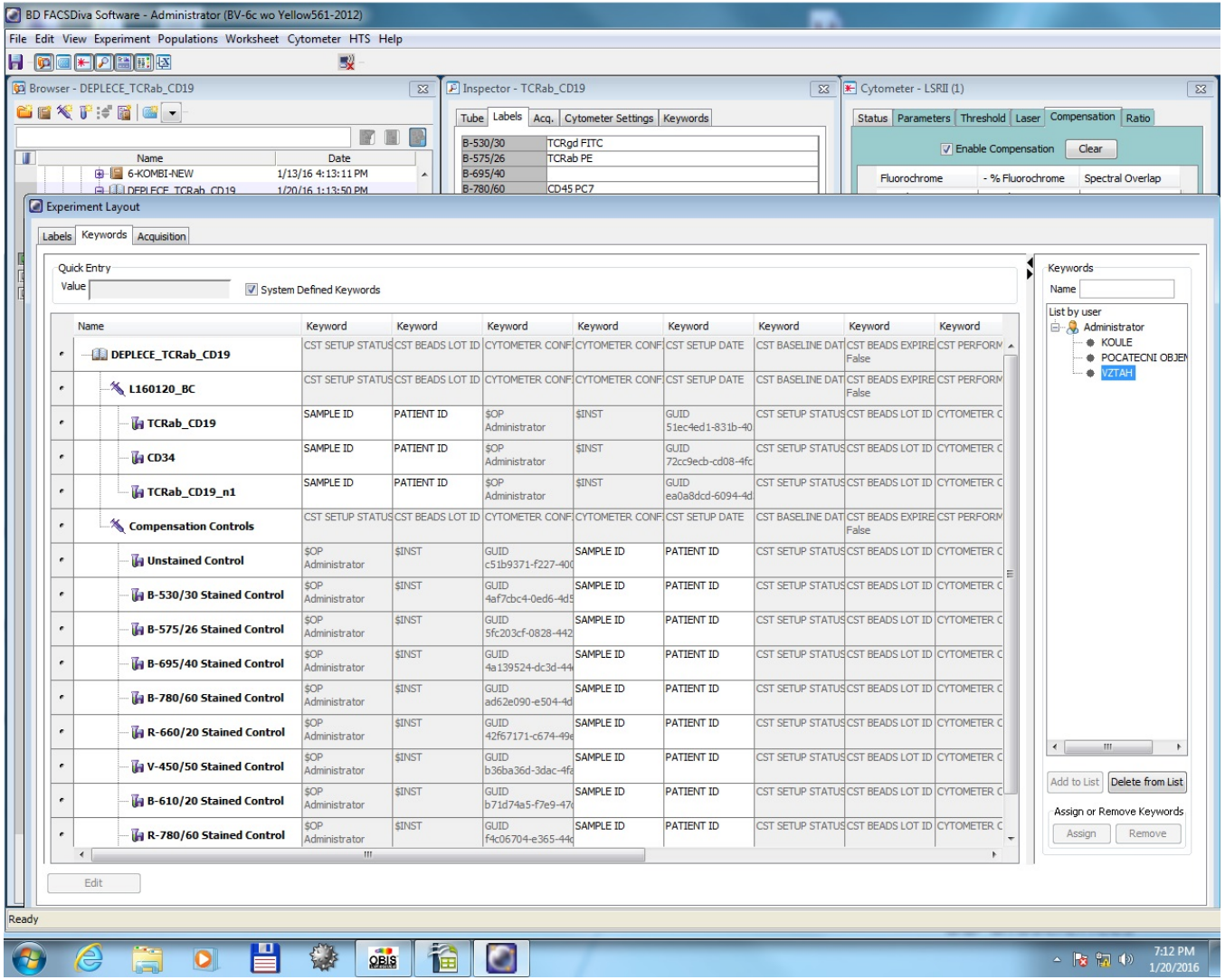
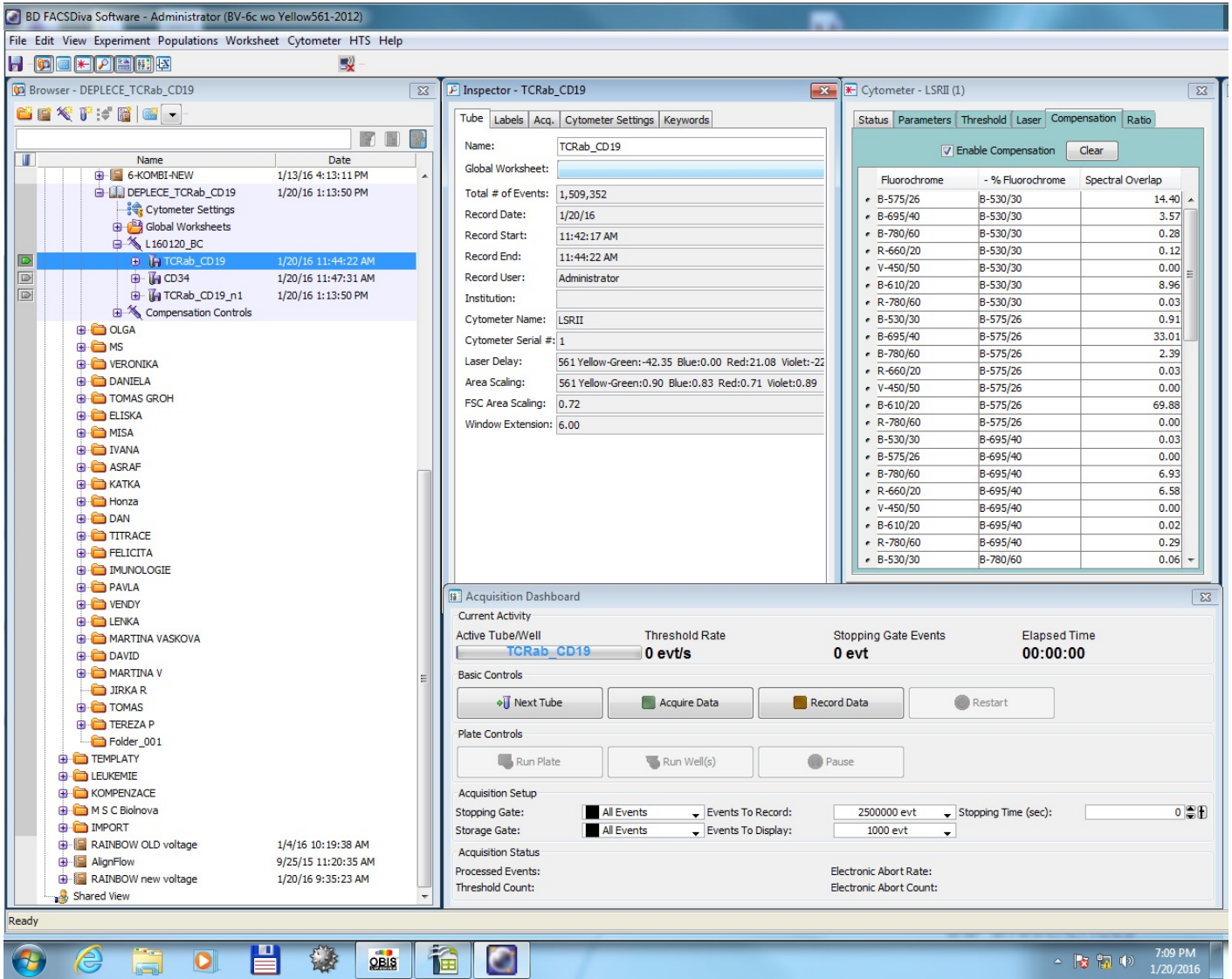
Clear
Fluorochrome - % Fluorochrome Spectral Overlap
B-575/26 B-530/30 14.40
B-695/40 B-530/30 3.57
B-780/60 B-530/30 0.28
R-660/20 B-530/30 0.12
V-450/50 B-530/30 0.00
Experiment Layout
Labels Keywords Acquisition
Quick Entry
Label
Name Label Label Label Label Label
L160120_BC
TCRab_CD19 B-530/30 TCRgd FITC B-575/26 TCRab PE B-695/40 B-780/60 CD45 PC7 R-660/20 CD3 APC
CD34 B-530/30 Syto16 B-575/26 CD3 PE B-695/40 CD45 PerCP B-780/60 R-660/20 CD34 APC
TCRab_CD19_n1 B-530/30 TCRgd FITC B-575/26 TCRab PE B-695/40 B-780/60 CD45 PC7 R-660/20 CD3 APC
Labels
Name
List by user
Administrator
BD Defined

SAMPLE ID / PATIENT ID

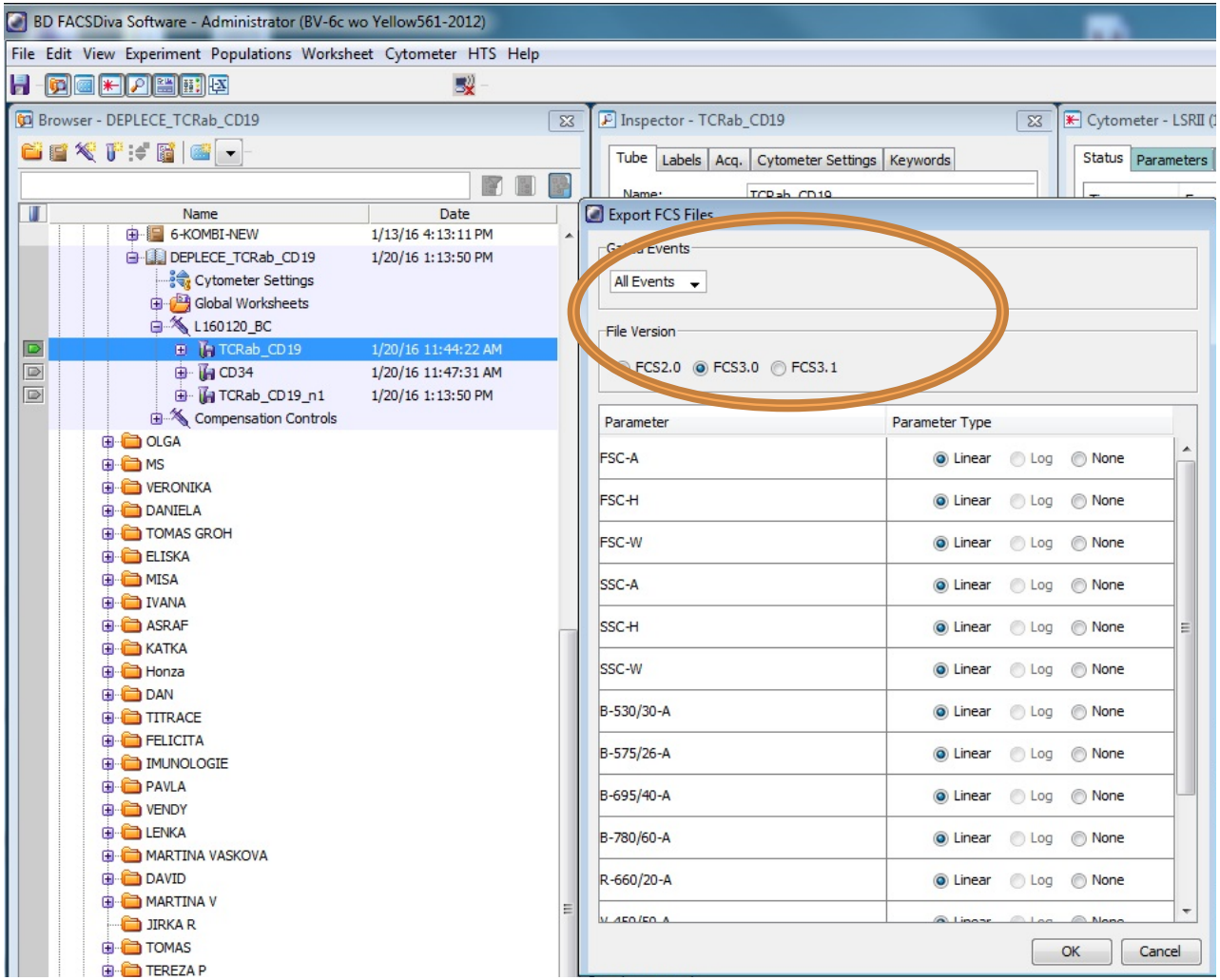


Compensation matrix



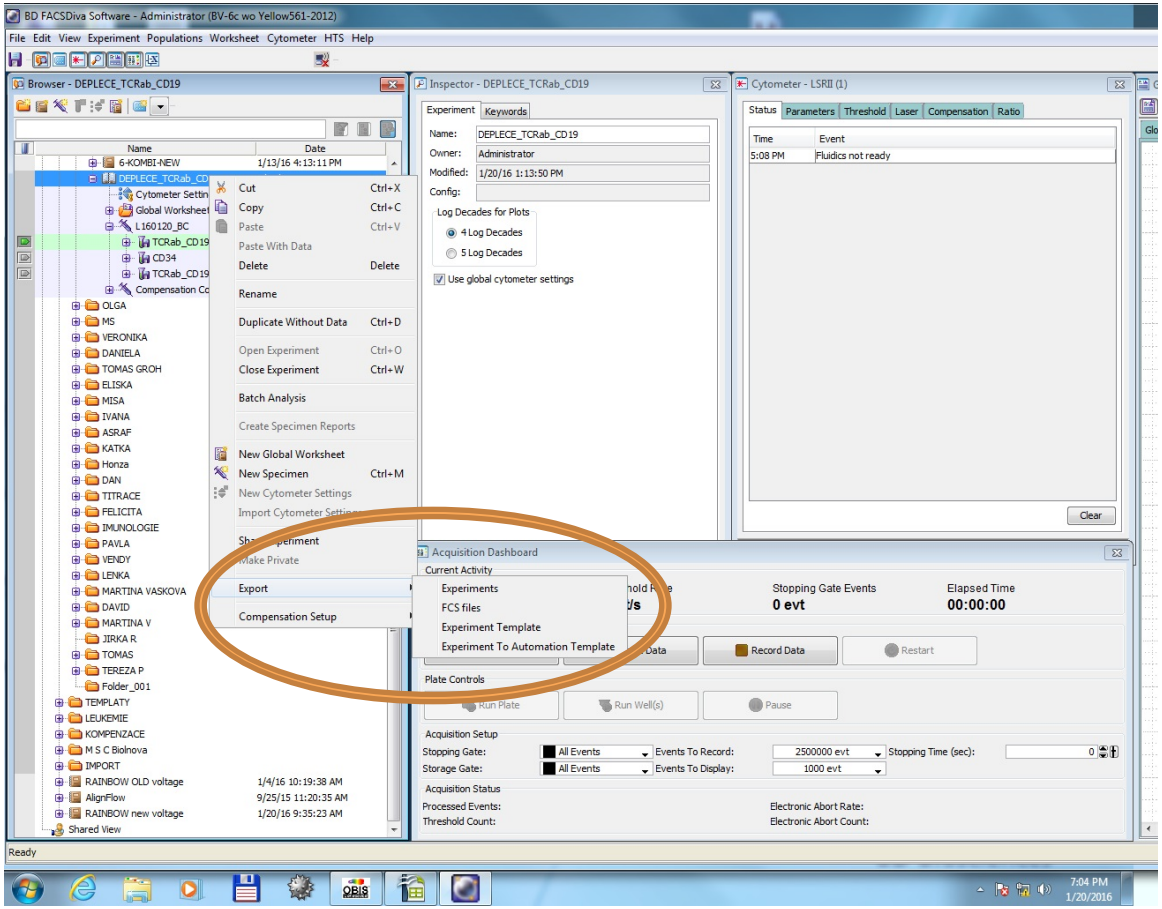
Export data as:

FCS 3.0



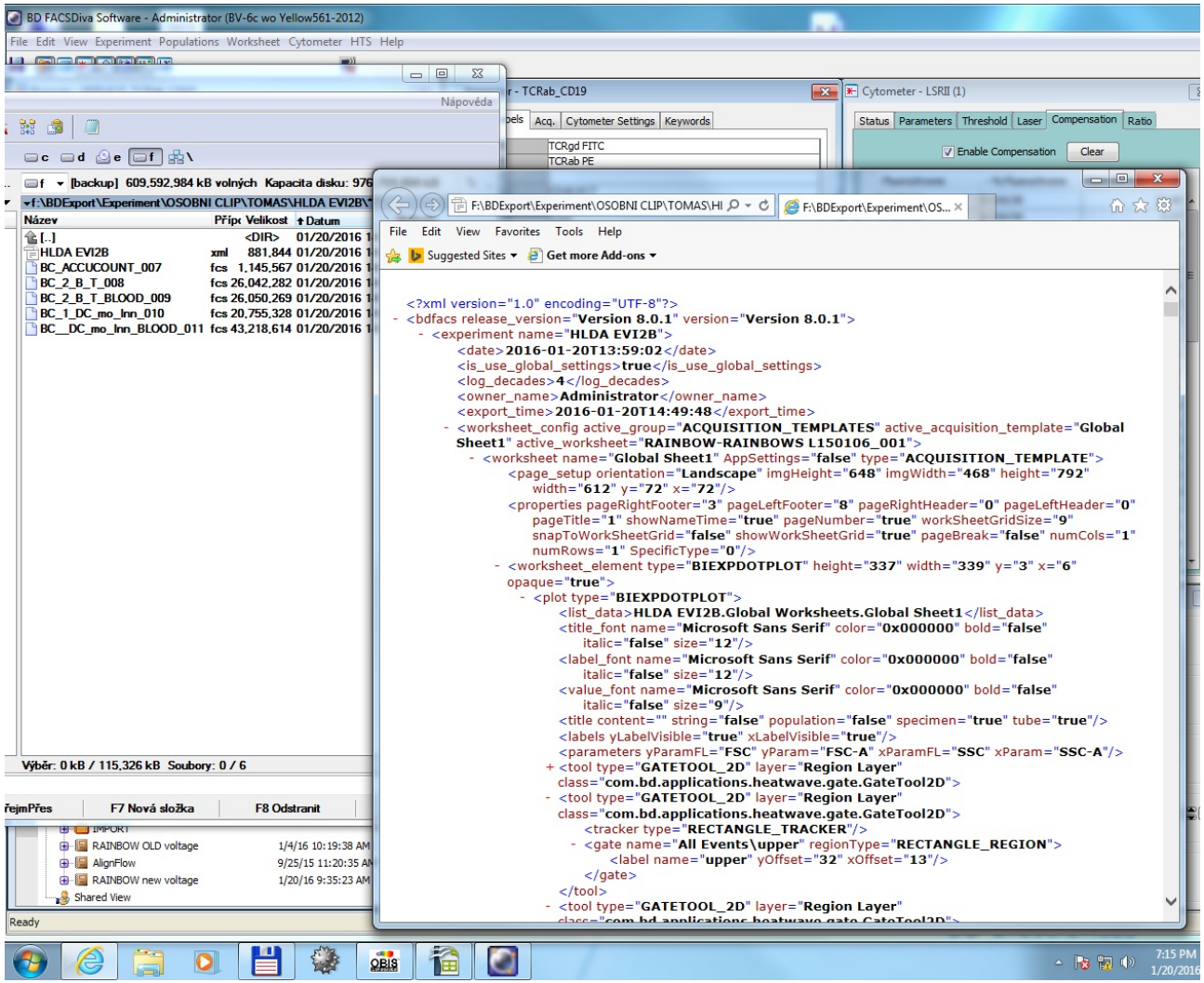
Other Diva formates

“experiment”
-> XML files



Ukládá veškeré informace o analýze (gaty, ploty atd) I o akvizici – templát pro experiment
Není ve formátu FCS 3 – chybí např vyplněná keywords

XML soubor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F8 Odstranit
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AlignFlow 9/25/15 11:20:35 AM
RAINBOW new voltage 1/20/16 9:35:23 AM
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TCRab PE
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7:15 PM
1/20/2016

Other formats: Navios/LMD

“LMD”
-> double FCS file

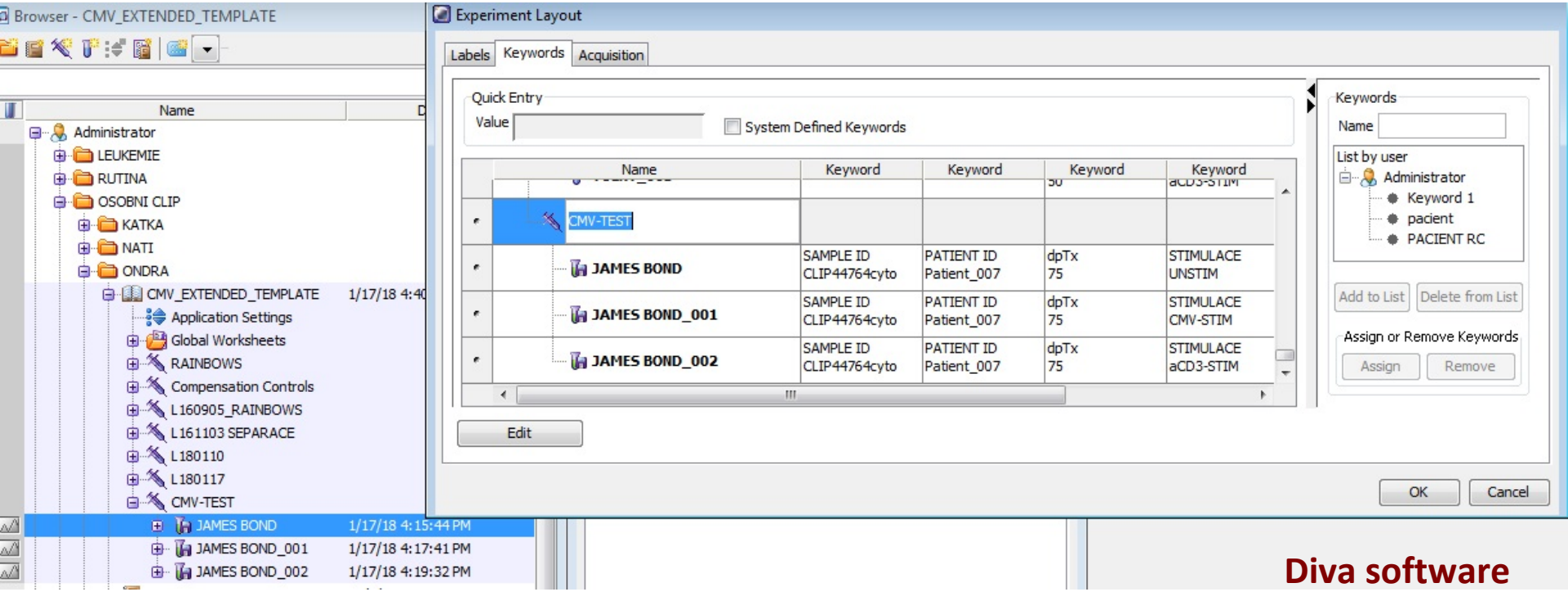
First piece:
FCS 2.0 file – simple, less resolution, fixed comp

Second piece:
FCS 3.0 file – full resolution data, without “keywords”

Annotation of FCS

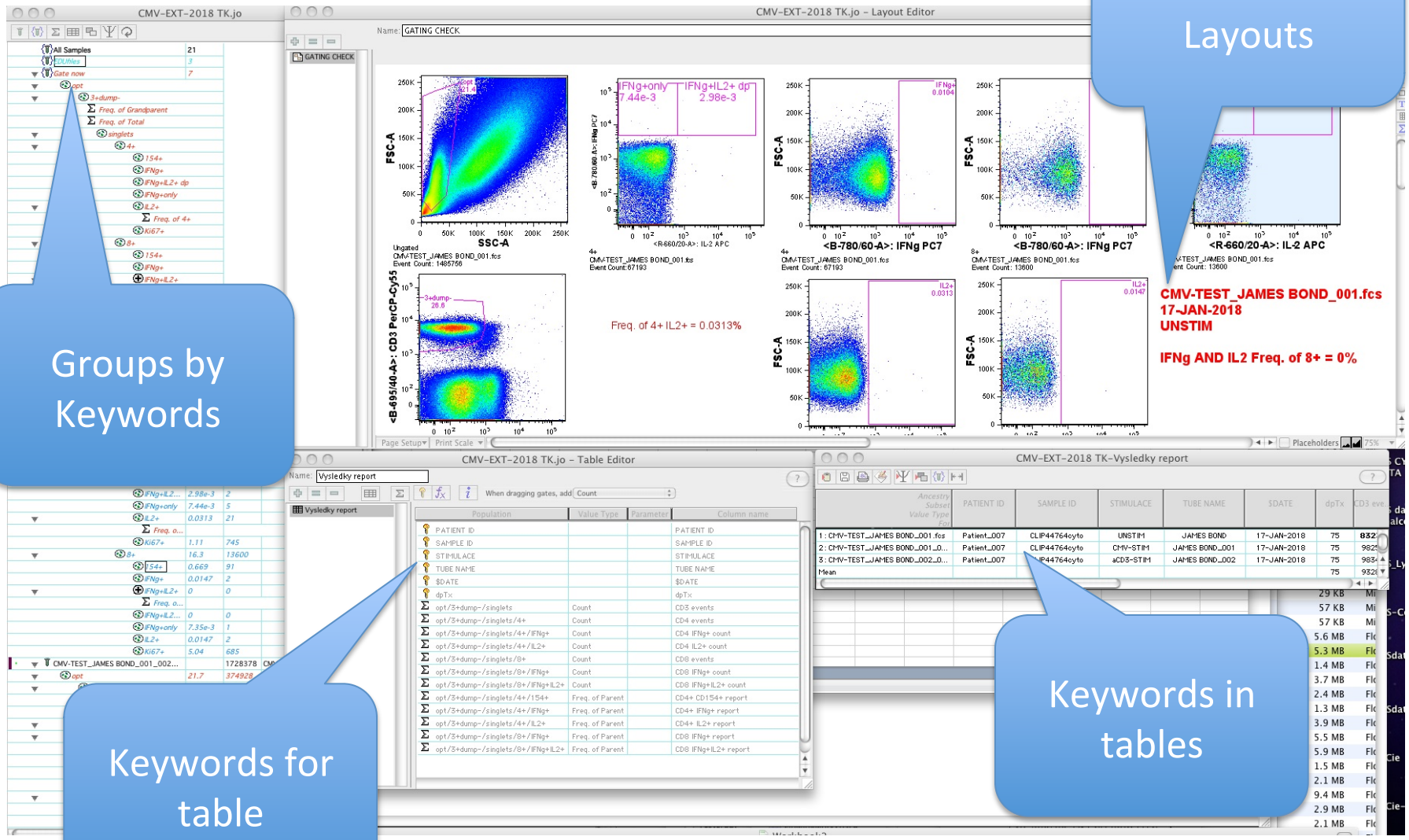
Keywords in datafiles:

Sample ID
Patient ID
Condition
Day post TX



Diva software

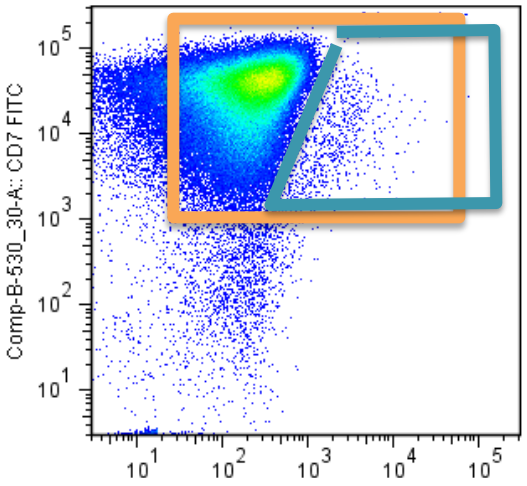
Annotations in Analysis



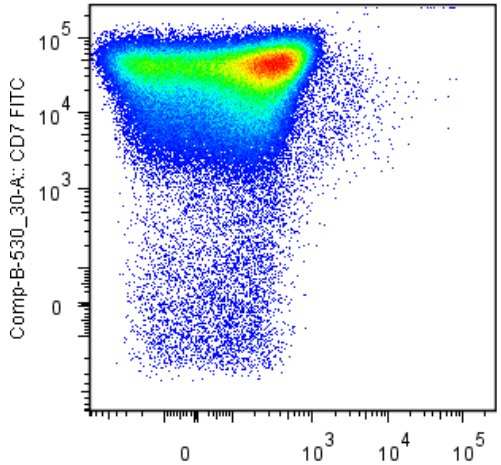
Flowjo software (Mac)

Quiz

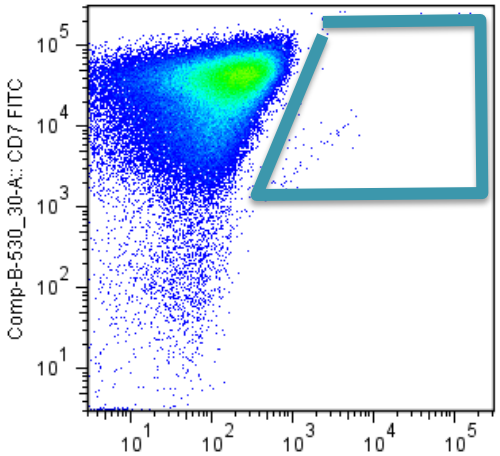
CD7 FITC



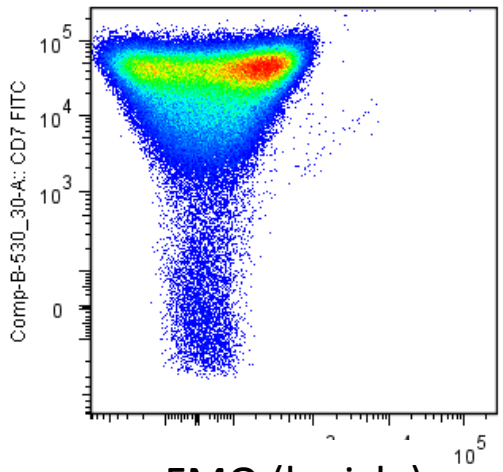
NG2 (logarithmic)



NG2 (logicle)



FMO (logarithmic)

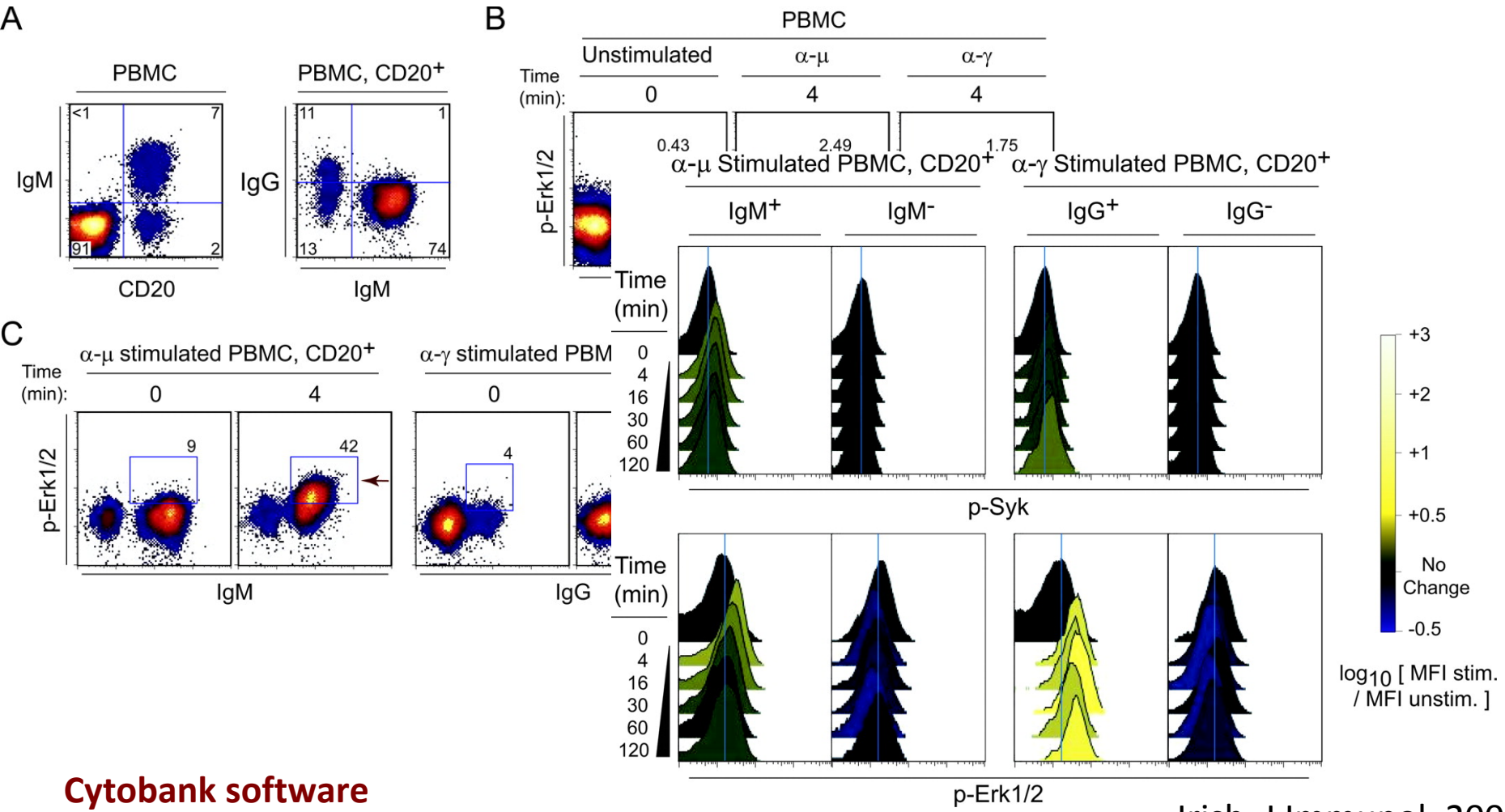


FMO (logicle)

Gating controls

- Isotype controls (sticky cells)
- FMO controls (multicolor, compensated data)
- Sample comparisons
  (stimulated, unstimulated)

Overview gating and histogram comparison



Cytobank software

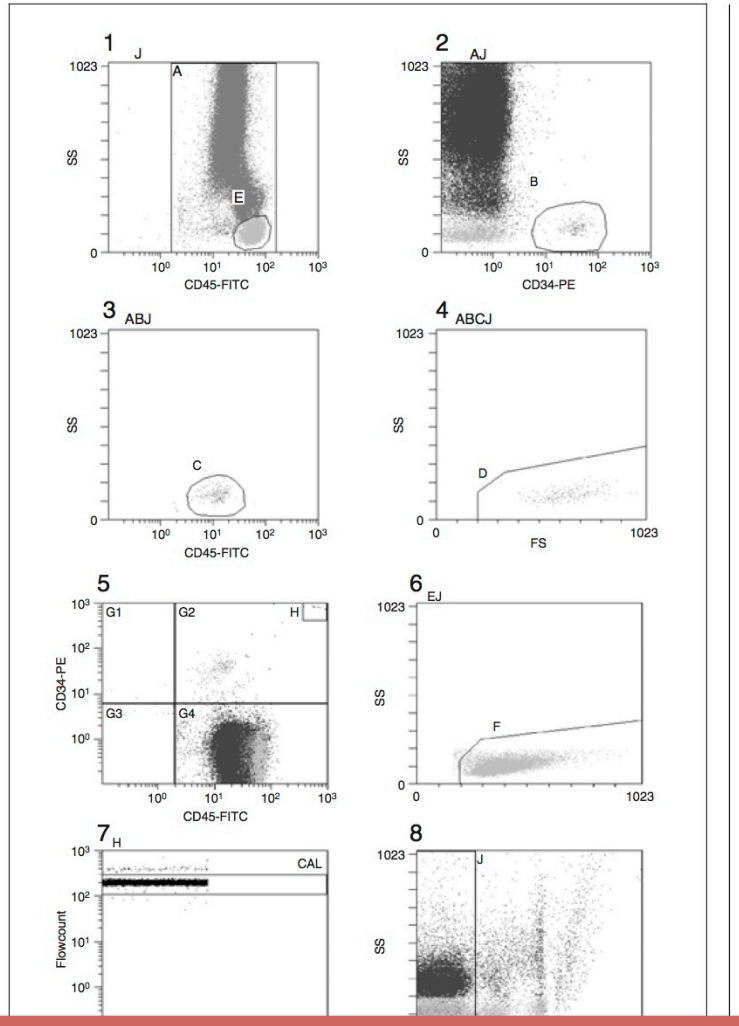
Irish, J Immunol. 2006

Bulk (batch analysis)
ISHAGE protocol CD34+ for HSCT

Aim:
Robust protocol for analysis (analyst independent)
Optimal gating (ISHAGE) and its visual control
Statistics – easy to export

Software:
FlowJo (Mac)

Gating strategy (copy, paste, apply to group)
Layout (gates)
Table (statistics)



Cytometry Part B (Clinical Cytometry) 00B:000–000 (2011)

Original Article

ISHAGE Protocol: Are We Doing It Correctly?

Alison Whitby,[1] Liam Whitby,[1] Matthew Fletcher,[1] John T. Reilly,[1] D. Robert Sutherland,[2] Michael Keeney,[3] and David Barnett[1]*

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[2]Department of Laboratory Hematology, University Health Network, Toronto, Canada

[3]Hematology/Flow Cytometry, London Health Sciences Centre, London, Canada

Background: Flow cytometric CD34$^+$ stem cell enumeration is routinely performed to optimize timing of peripheral blood stem cell collections and assess engraftment capability of the apheresis product. While a number of different flow methodologies have been described, the highly standardized ISHAGE protocol is currently the most widely employed, with 204/255 (81%) international participants in the UK NEQAS CD34$^+$ stem cell enumeration program indicating their use of this method. Recently, two laboratories were identified as persistent poor performers, a fact attributed to incorrect ISHAGE protocol usage/setup. This prompted UK NEQAS to question whether other laboratories were making similar errors and, if so, how this might affect individual EQA performance.

Methods and Results: In send out 0801, where two stabilized samples were issued, the EQA center surveyed 255 participants with flow analysis data and subsequent results collected. One hundred and ninety-six laboratories returned results with 103 returning dot plots. Eighty-three out of one hundred and three stated that they used the ISHAGE protocol gating strategy but 43% (36/83) were incorrectly set-up. Analysis of the data showed those incorrectly using single platform ISHAGE gating strategy were twice as likely to fail an EQA exercise compared to those using the protocol correctly. This failure rate increased two fold when incorrect ISHAGE protocol was used in a dual platform setting.

Conclusion: This study suggests a widespread fundamental lack of understanding of the ISHAGE protocol and the need to deploy it correctly, potentially having significant clinical implications and highlights the need to monitor participants rigorously in their deployment of the ISHAGE protocol. It is hoped that once these findings have been disseminated, performance can be improved. © 2011 International Clinical Cytometry Society

Key terms: CD34; stem cells; ISHAGE; quality control; quality assessment; EQA

How to cite this article: Whitby A, Whitby L, Fletcher M, Reilly JT, Sutherland DR, Keeney M, Barnett D. ISHAGE protocol: Are we doing it correctly? Cytometry Part B 2011; 00B: 000–000.

Over the last 15 years or so, cytokine-mobilized peripheral blood stem cells (PBSC) have largely replaced bone marrow as a source of hematopoietic stem cells (HSCs) in the majority of autologous and (in an increasing proportion) allogeneic PBSC transplants (1,2). The HSCs in marrow and peripheral blood, which are responsible for multilineage engraftment in the transplant setting express the cell surface marker CD34 (3,4). Flow cytometric enumeration of CD34$^+$ cells provides a rapid means of measuring this clinically useful surrogate marker of graft adequacy in all sources of HSCs and weight. In addition to determining yield, the number of CD34$^+$ cells mobilized to the peripheral blood is also a predictor of the success of apheresis (5) and can be used to monitor, "on-line," the yield of CD34$^+$ cells (6).

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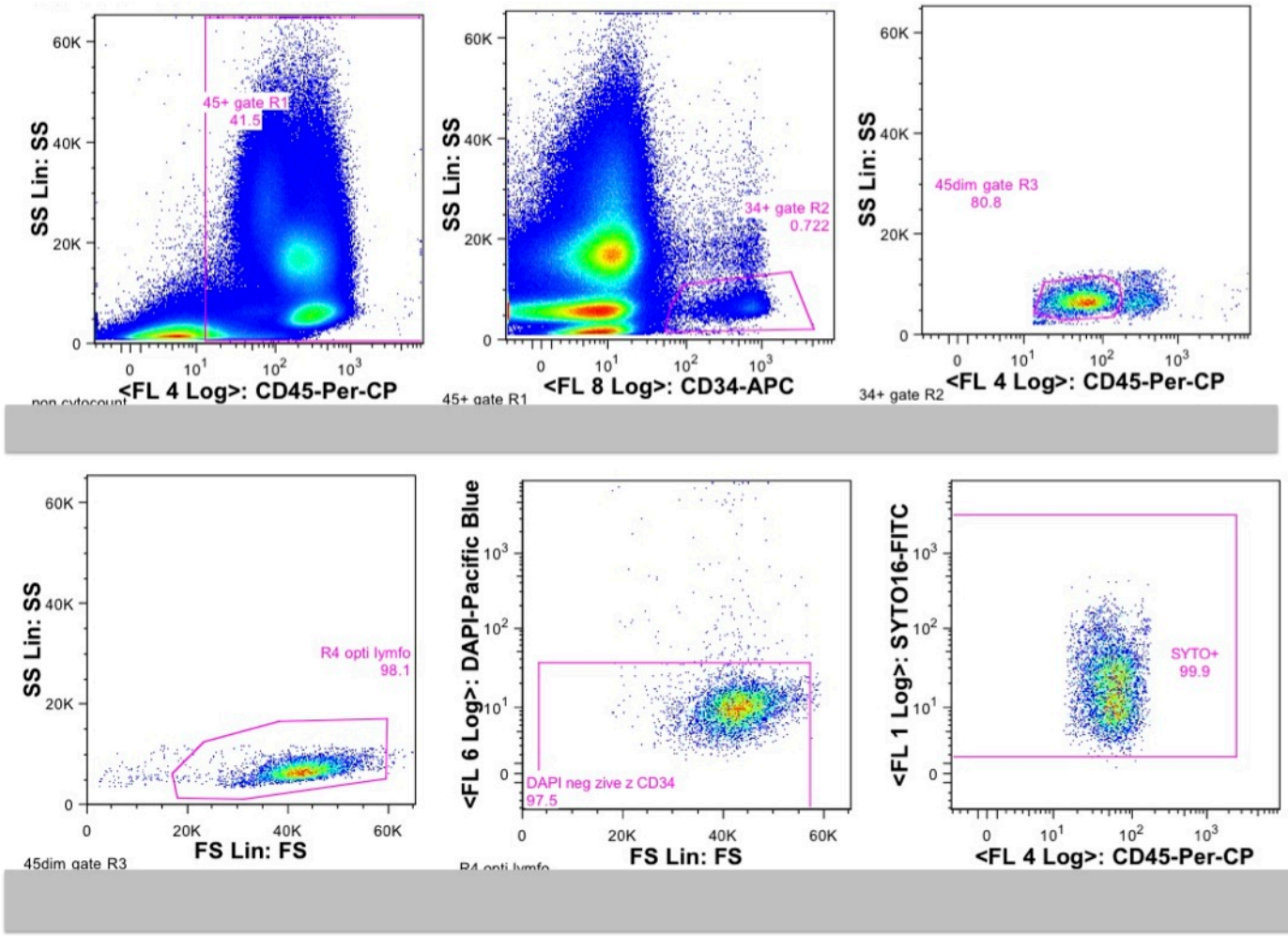
increased two fold when incorrect ISHAGE protocol was used in a dual platform setting.

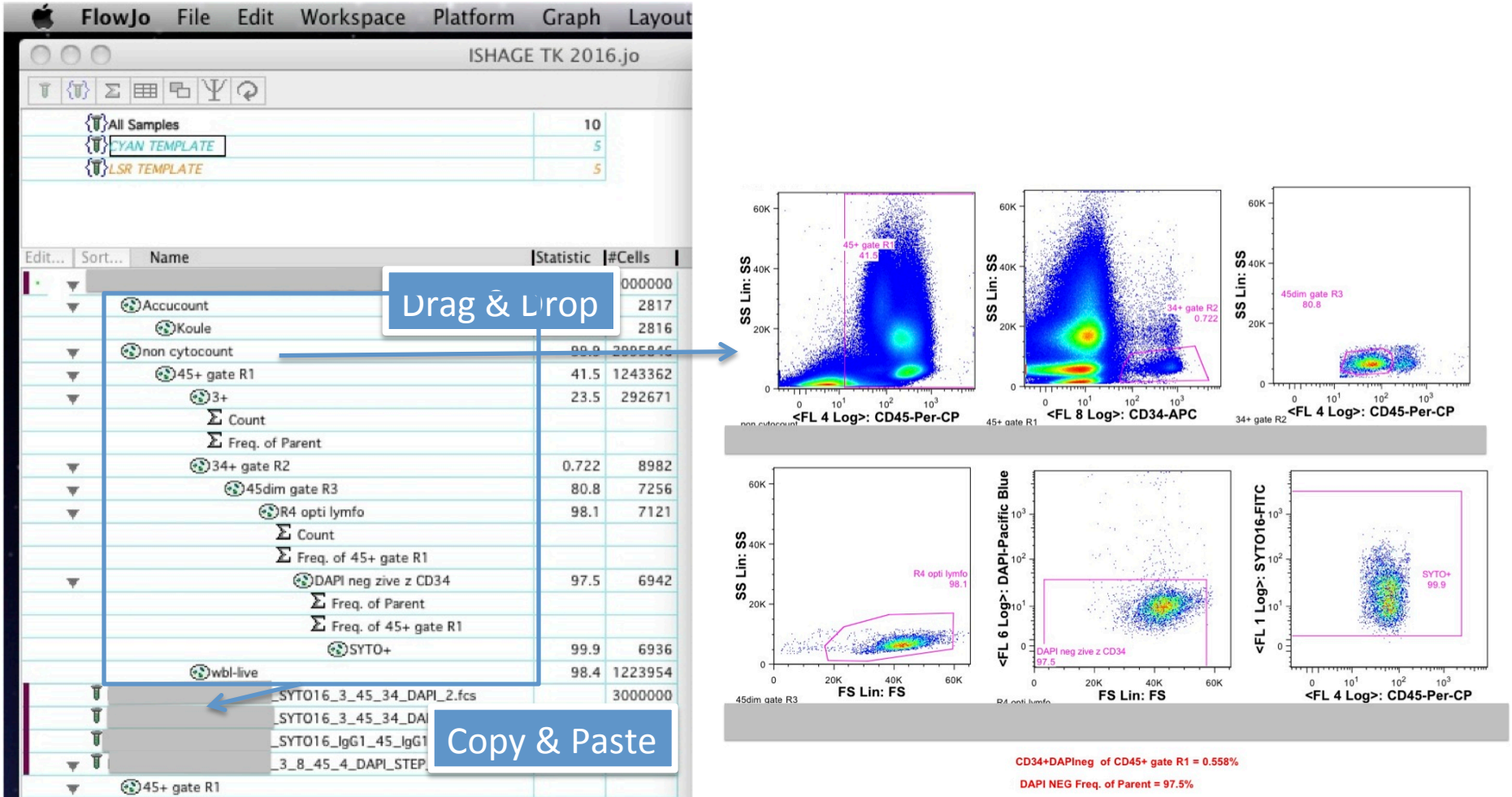
Conclusion: This study suggests a widespread fundamental lack of understanding of the ISHAGE protocol and the need to deploy it correctly, potentially having significant clinical implications and highlights the need to monitor participants rigorously in their deployment of the ISHAGE protocol. It is hoped that once these findings have been disseminated, performance can be improved. © 2011 International Clinical Cytometry Society

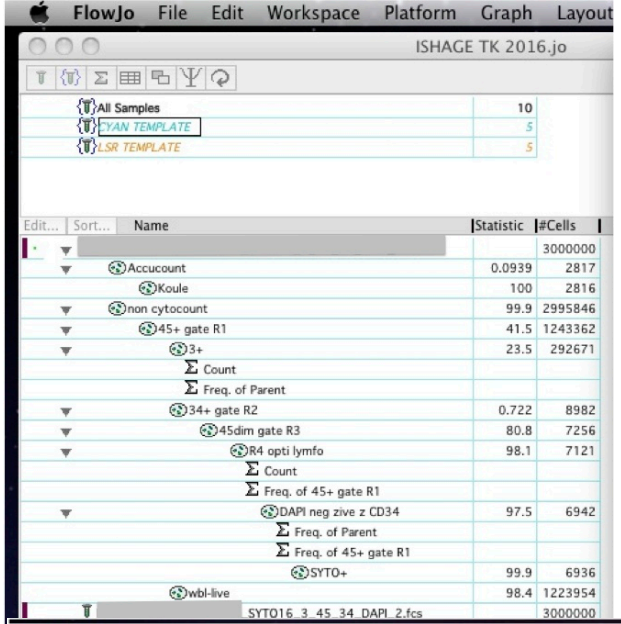
ISHAGE protocol
Layout in FlowJo



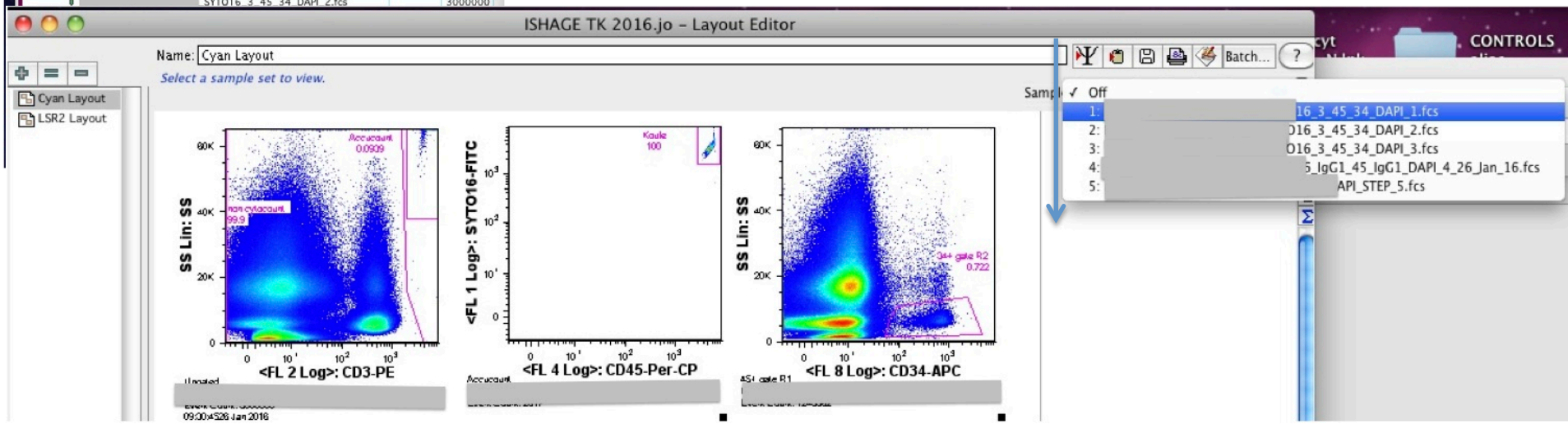
CD34+DAPIneg of CD45+ gate R1 = 0.558%

DAPI NEG Freq. of Parent = 97.5%


FlowJo File Edit Workspace Platform Graph Layout
ISHAGE TK 2016.jo
All Samples 10
CYAN TEMPLATE 5
LSR TEMPLATE 5
Edit... Sort... Name Statistic #Cells
Accucount 2817
Koule 2816
non cytocount
45+ gate R1 41.5 1243362
3+ 23.5 292671
Count
Freq. of Parent
34+ gate R2 0.722 8982
45dim gate R3 80.8 7256
R4 opti lymfo 98.1 7121
Count
Freq. of 45+ gate R1
DAPI neg zive z CD34 97.5 6942
Freq. of Parent
Freq. of 45+ gate R1
SYTO+ 99.9 6936
wbl-live 98.4 1223954
_SYTO16_3_45_34_DAPI_2.fcs 3000000
_SYTO16_3_45_34_DA
_SYTO16_IgG1_45_IgG1
_3_8_45_4_DAPI_STEP
45+ gate R1
3+
dapi-
4+
8+
8+
Drag & Drop
Copy & Paste
SS Lin: SS
<FL 4 Log>: CD45-Per-CP
<FL 8 Log>: CD34-APC
<FL 6 Log>: DAPI-Pacific Blue
<FL 1 Log>: SYTO16-FITC
FS Lin: FS
CD34+DAPIneg of CD45+ gate R1 = 0.558%
DAPI NEG Freq. of Parent = 97.5%

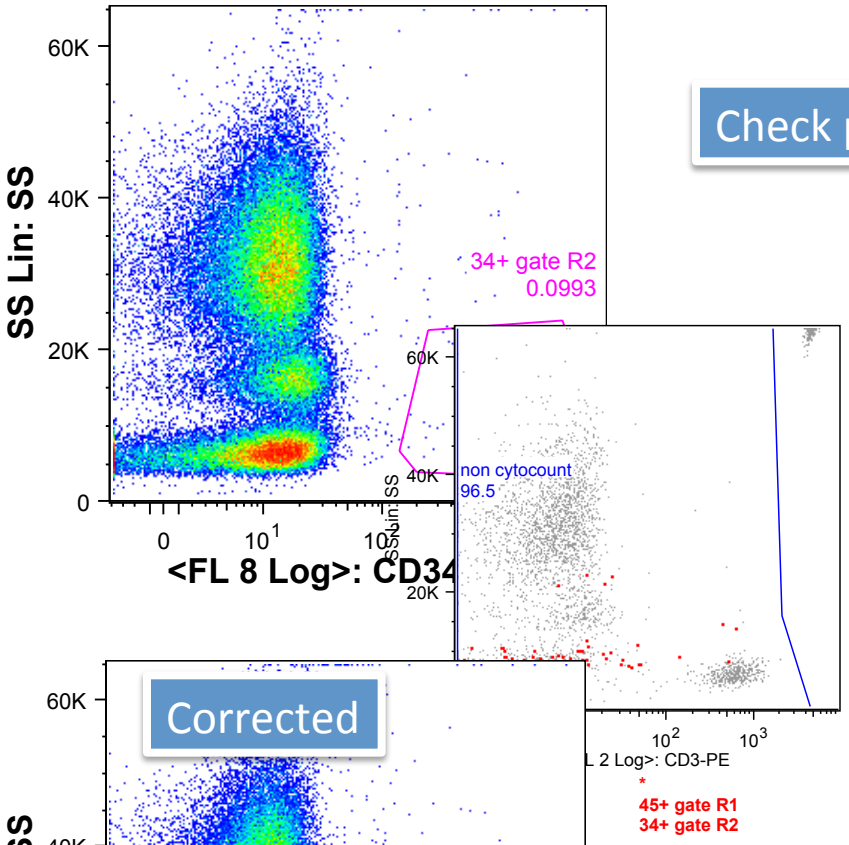


Scroll down

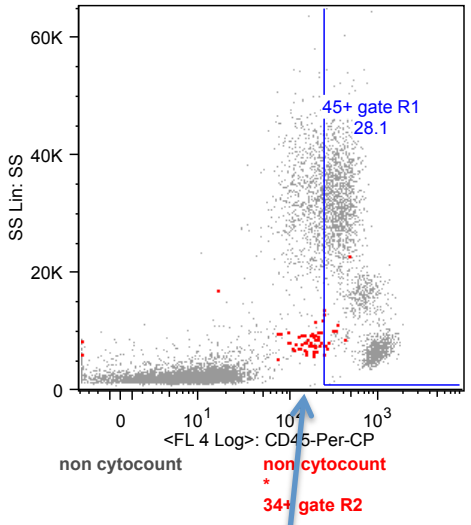


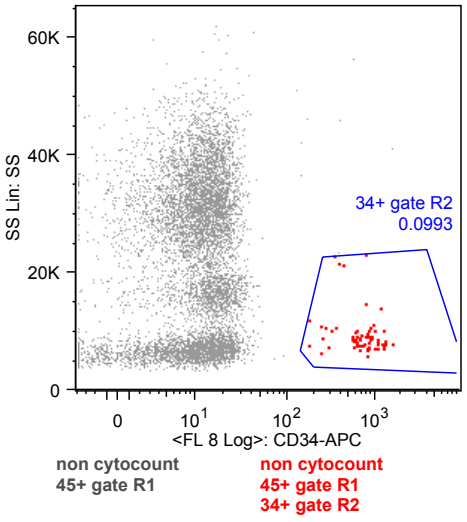
Adjust if needed

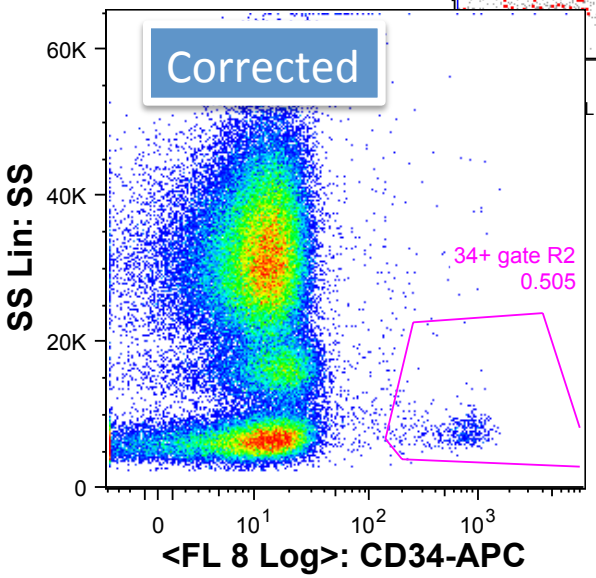
Backgating



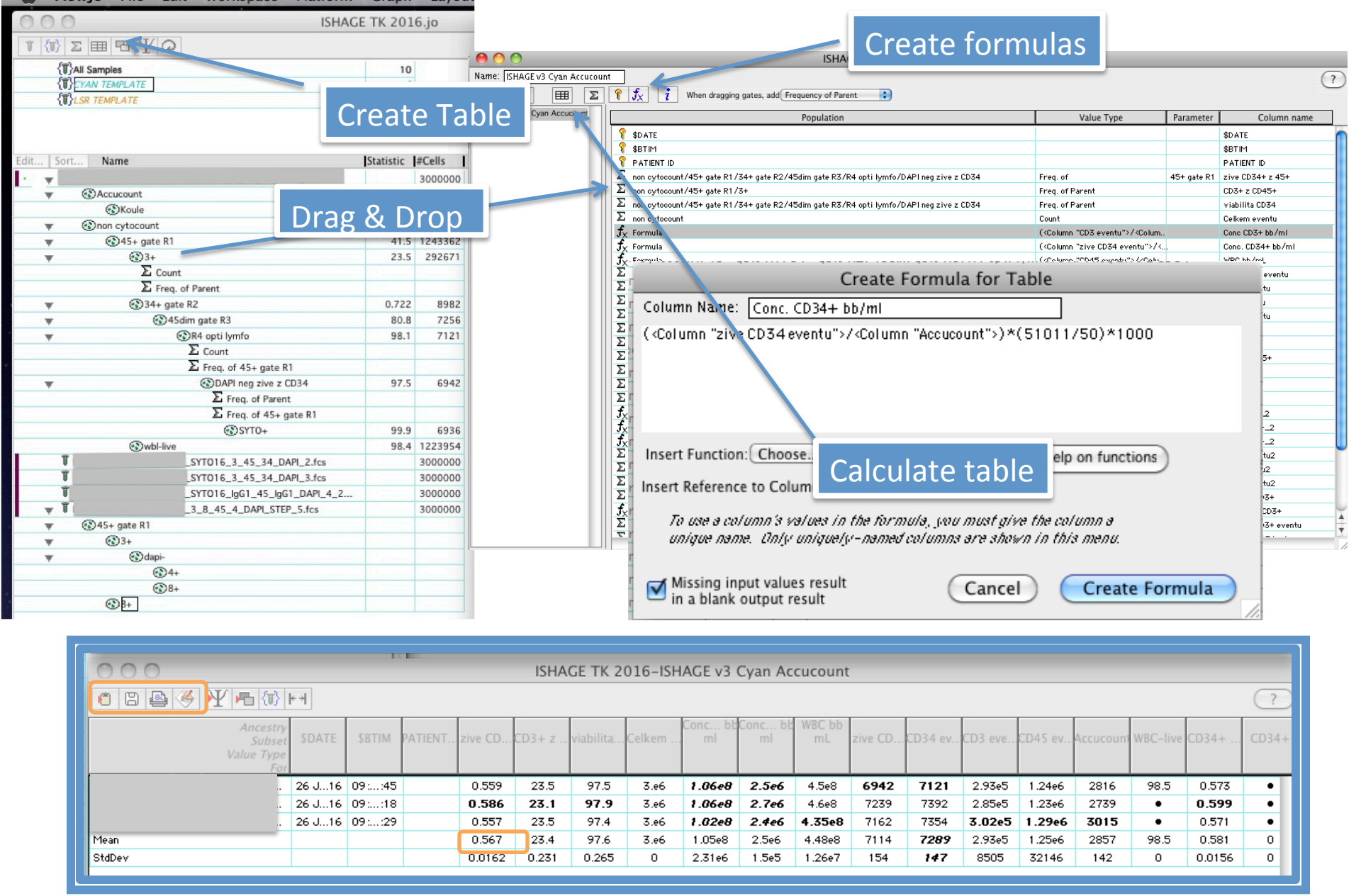
Check parental gates by backgating







Wrong position of CD45+ gate!!! by backgating



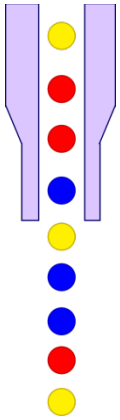
Ancestry / Subset / Value Type / For	$DATE	$BTIM	PATIENT...	zive CD...	CD3+ z ...	viabilita...	Celkem ...	Conc... bb ml	Conc... bb ml	WBC bb mL	zive CD...	CD34 ev...	CD3 eve...	CD45 ev...	Accucount	WBC-live	CD34+ ...	CD34+
	26 J...16	09:...:45		0.559	23.5	97.5	3.e6	***1.06e8***	***2.5e6***	4.5e8	**6942**	**7121**	2.93e5	1.24e6	2816	98.5	0.573	•
	26 J...16	09:...:18		**0.586**	**23.1**	**97.9**	3.e6	***1.06e8***	***2.7e6***	4.6e8	7239	7392	2.85e5	1.23e6	2739	•	**0.599**	•
	26 J...16	09:...:29		0.557	23.5	97.4	3.e6	***1.02e8***	***2.4e6***	**4.35e8**	7162	7354	**3.02e5**	**1.29e6**	**3015**	•	0.571	•
Mean				0.567	23.4	97.6	3.e6	1.05e8	2.5e6	4.48e8	7114	***7289***	2.93e5	1.25e6	2857	98.5	0.581	0
StdDev				0.0162	0.231	0.265	0	2.31e6	1.5e5	1.26e7	154	***147***	8505	32146	142	0	0.0156	0

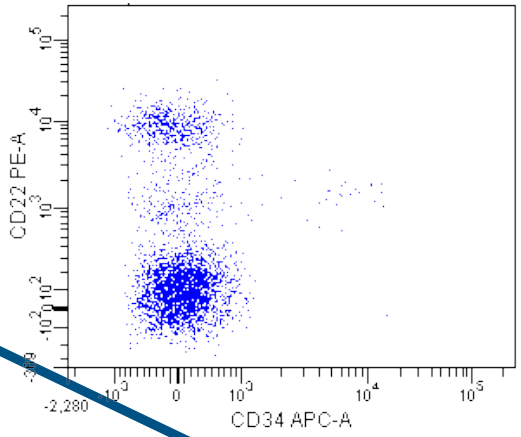
Curse of dimensionality

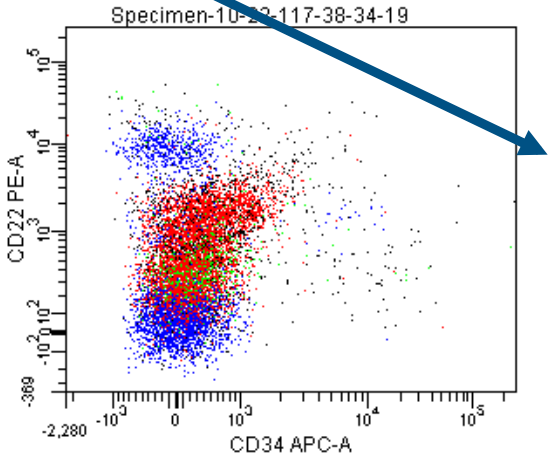
More parameters => more information

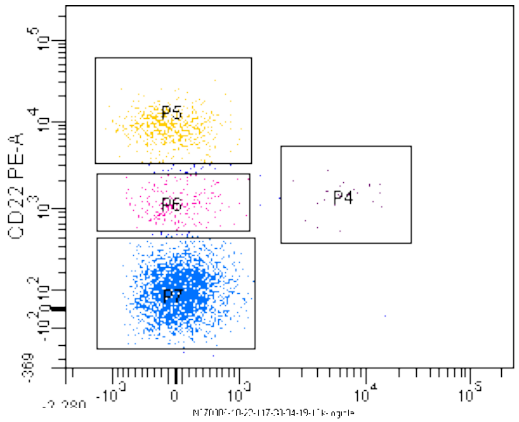
➔ More difficult to understand it

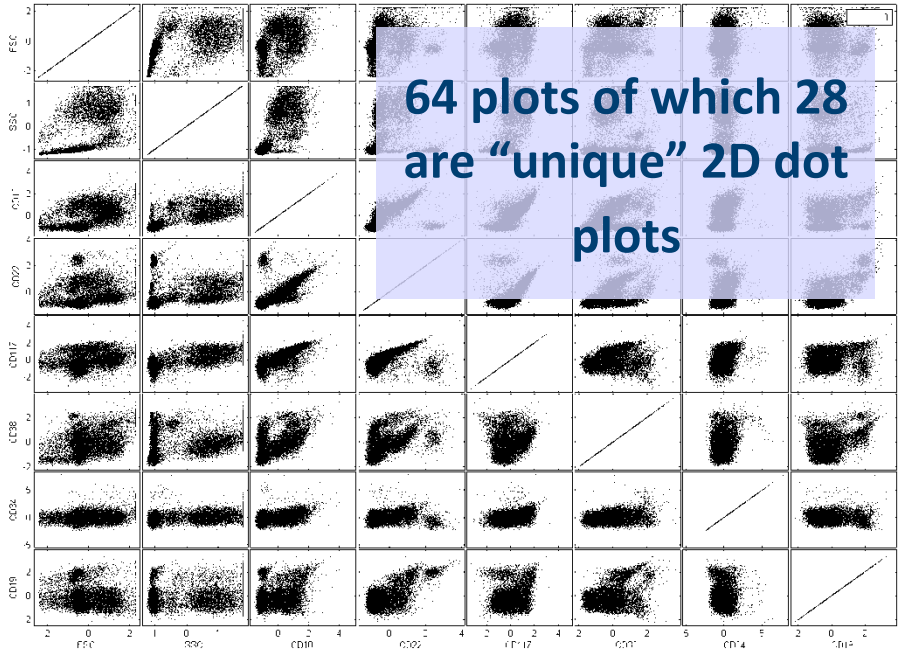
8-color data – perplexed!











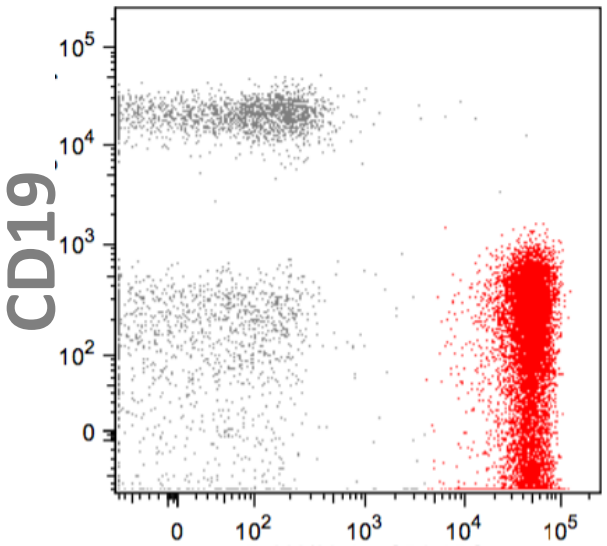
64 plots of which 28 are “unique” 2D dot plots

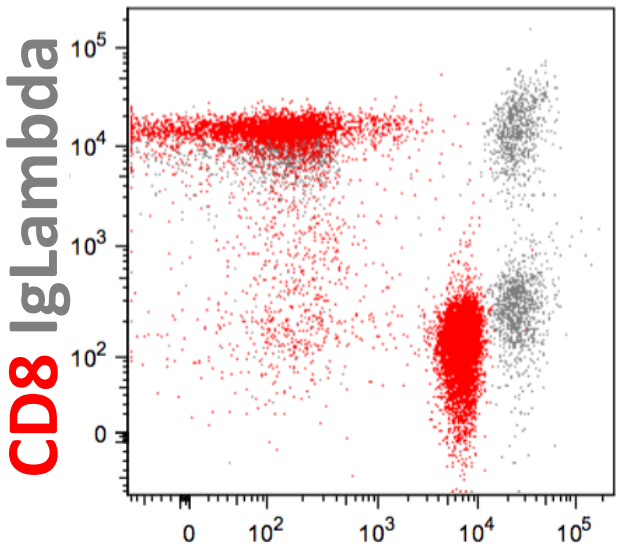
Slide Karel Fišer

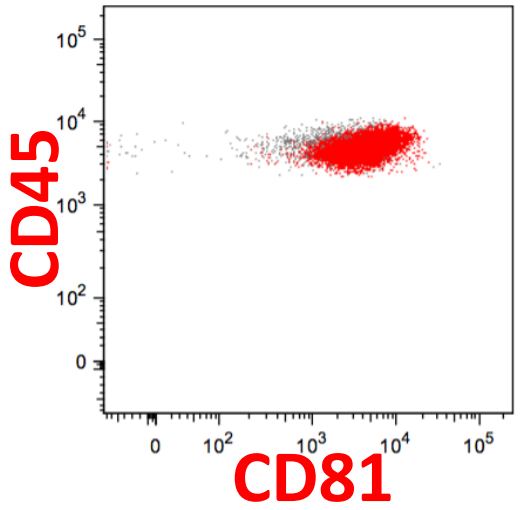
Projection of n-dimensions to 2D space

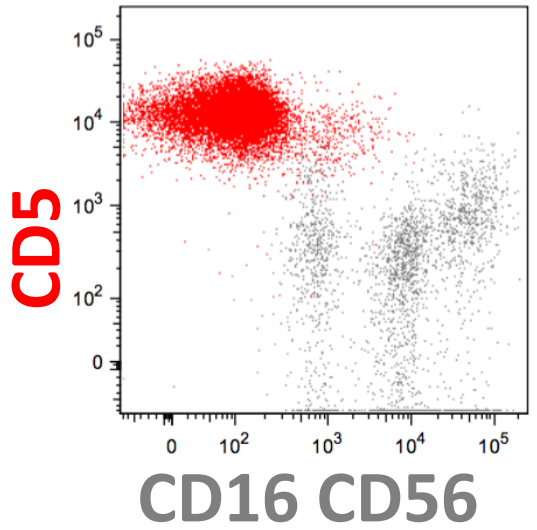
Lymphocytes

8 color data



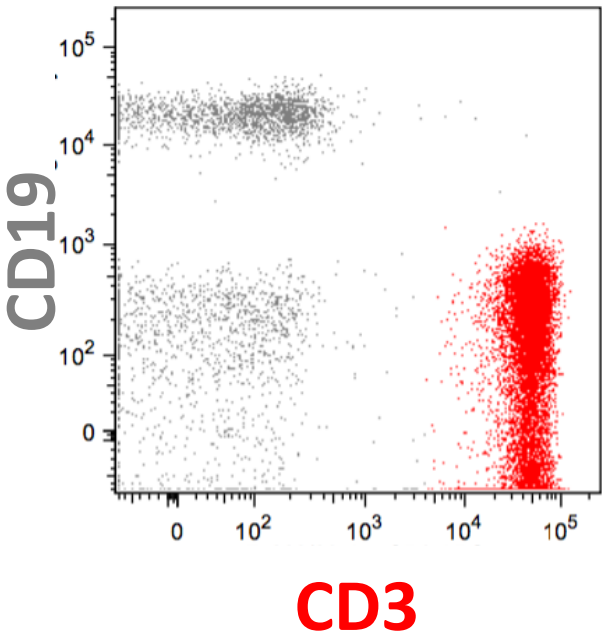




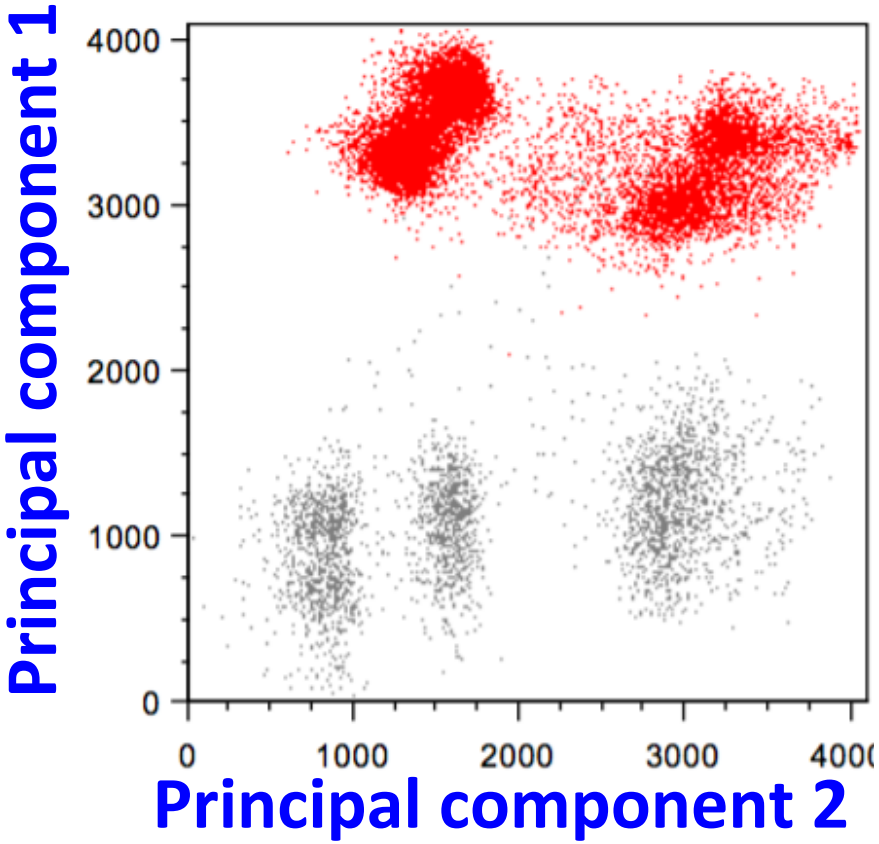


Principal component analysis

Lymphocytes

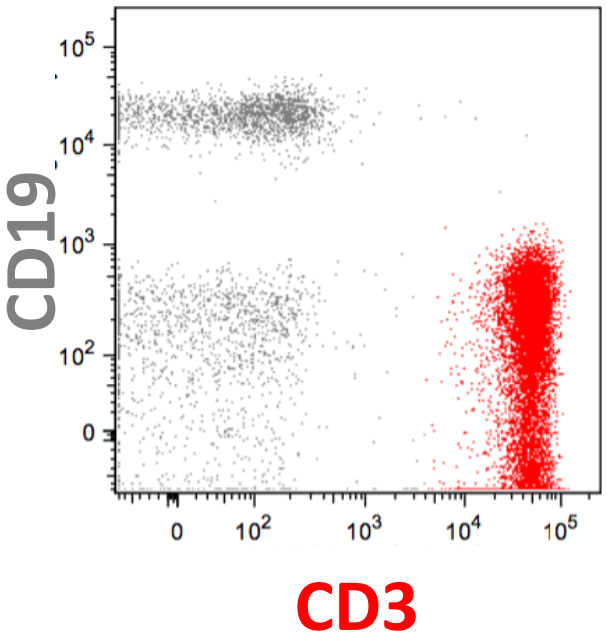


8 color data

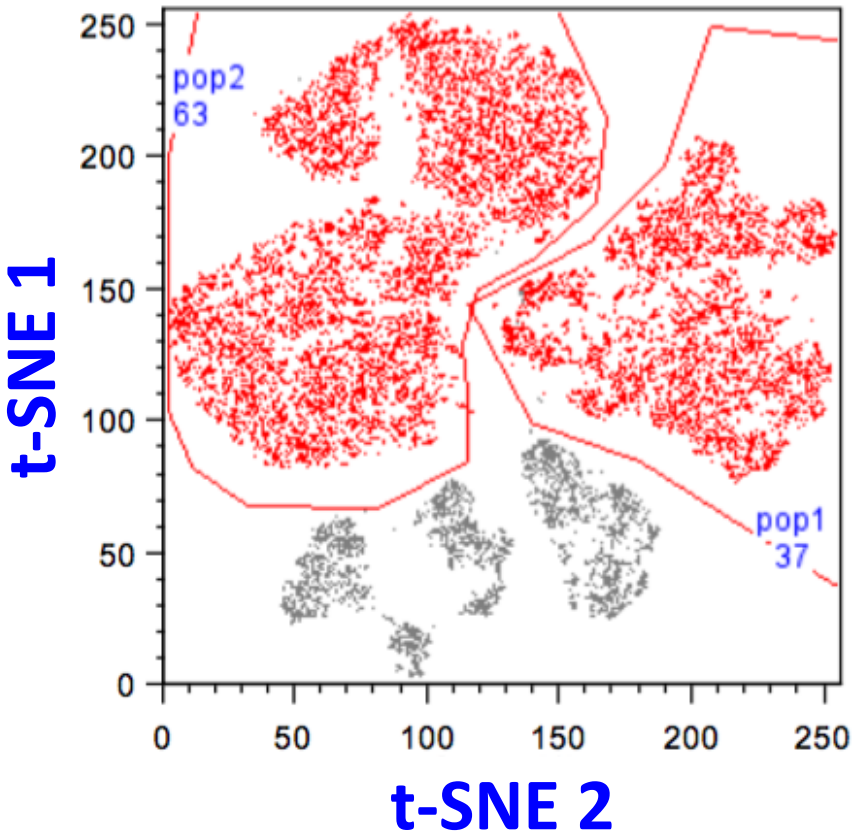


t-Distributed Stochastic Neighbor Embedding (t-SNE)

Lymphocytes



8 color data

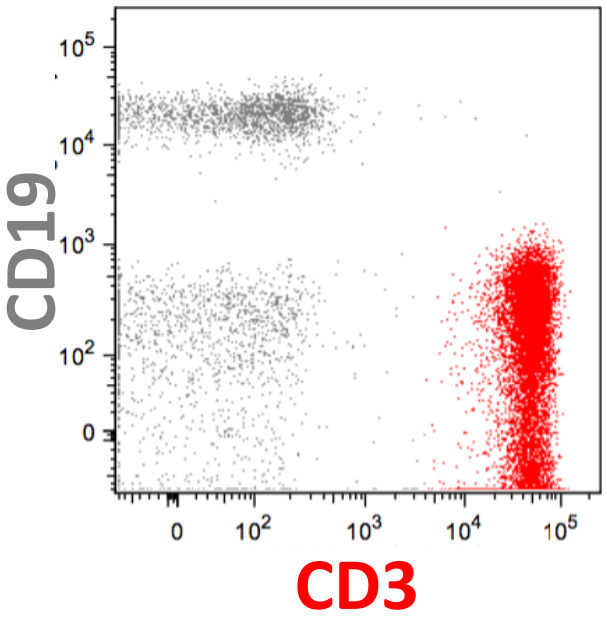


Flowjo software (Mac)

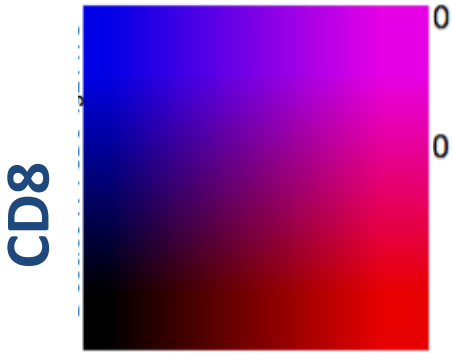
t-Distributed Stochastic Neighbor Embedding (t-SNE)

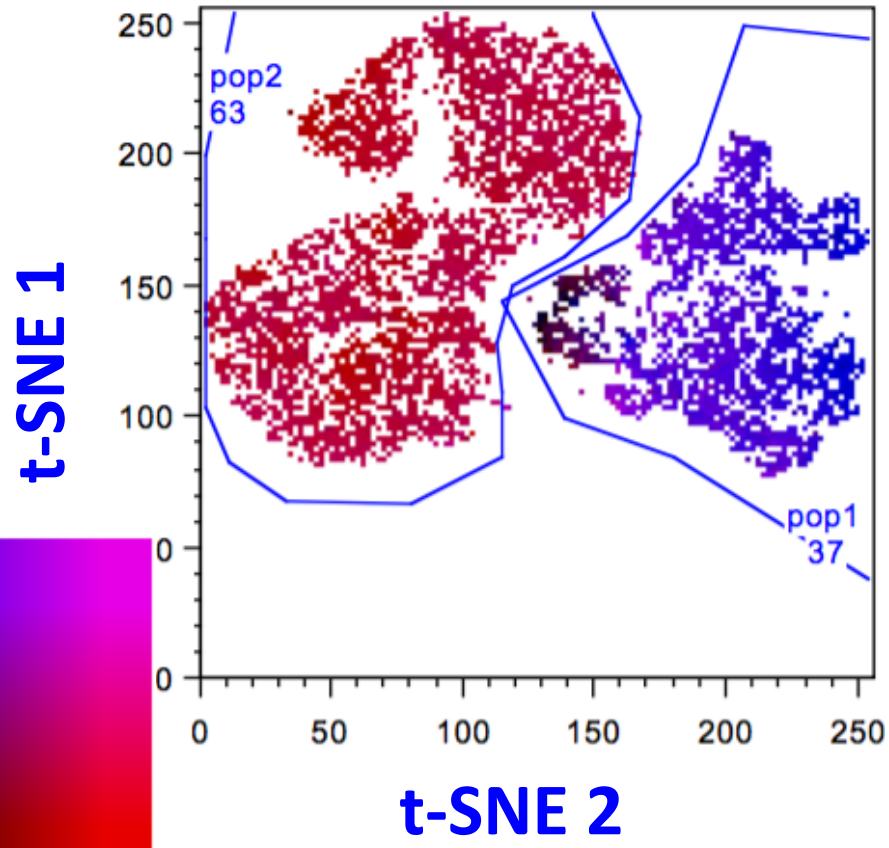
Lymphocytes

8 color data



Parameter as color:





Flowjo software (Mac)

LEUKEMIA (t-SNE)

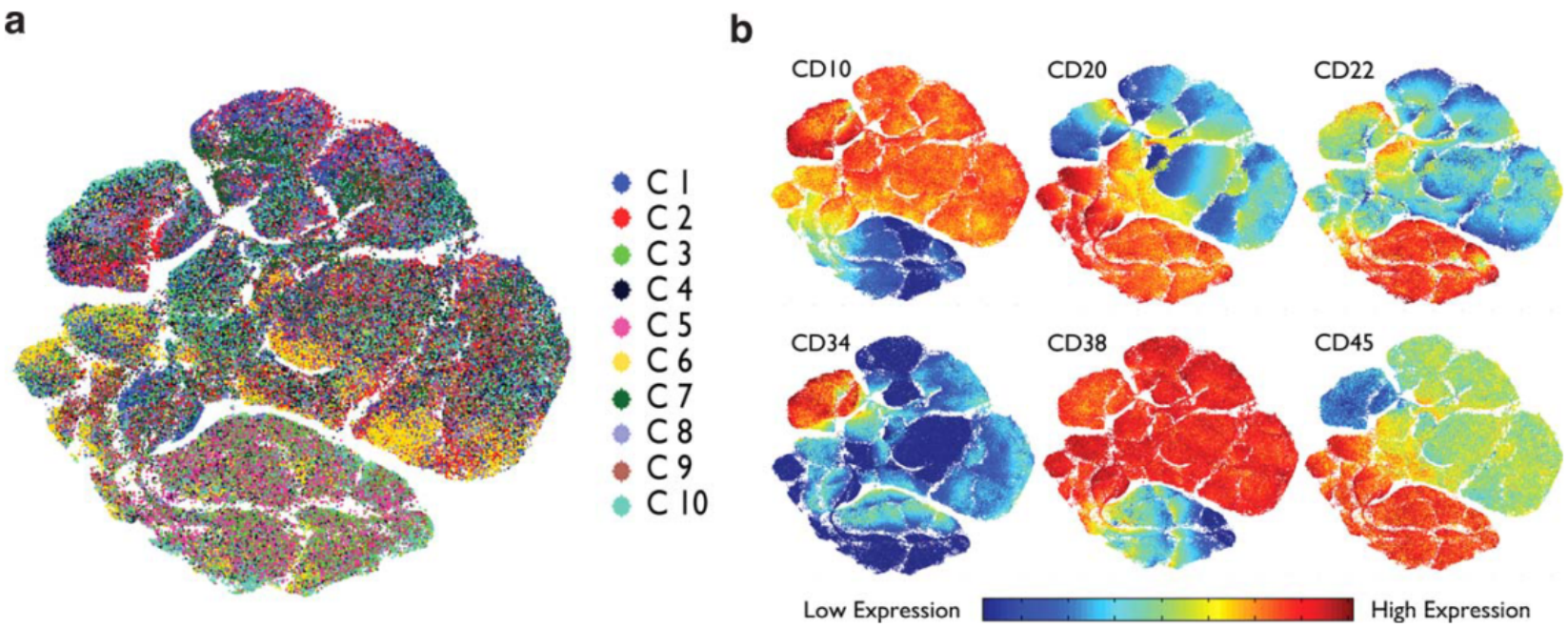
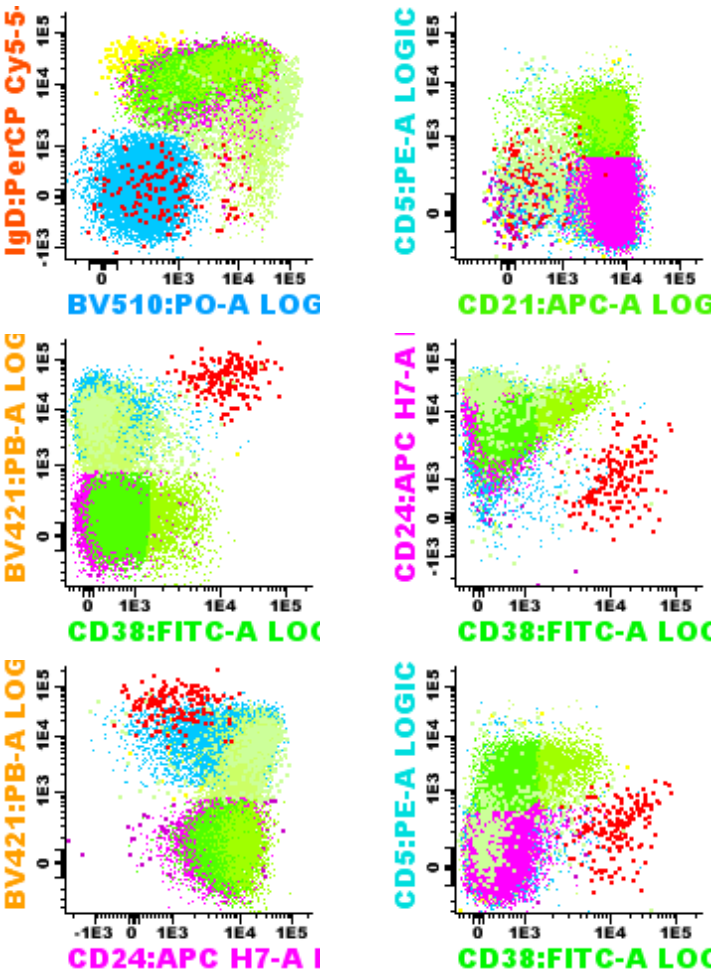


FIG. 1. viSNE map of normal bone marrow B cells labeled with a single 8-color antibody combination (tube A). Panel **A**: Each of the 10 control bone marrow samples used to construct the map (C1 through C10) is identified individually with a unique color. Each point in the viSNE map represents an individual cell colored by sample identity. Panel **B**: viSNE map shown in Panel (A), in which each cell is colored to reflect intensity of antigen expression for six different markers in tube A. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DiGiuseppe, J. a, Tadmor, M. D. & Pe'er, D. Detection of minimal residual disease in B lymphoblastic leukemia using viSNE. Cytom. Part B Clin. Cytom. 88, 294–304 (2015).

B cells' subsets (t-SNE)



Infinicyt software

IMMATURE

NAIVE CELLS

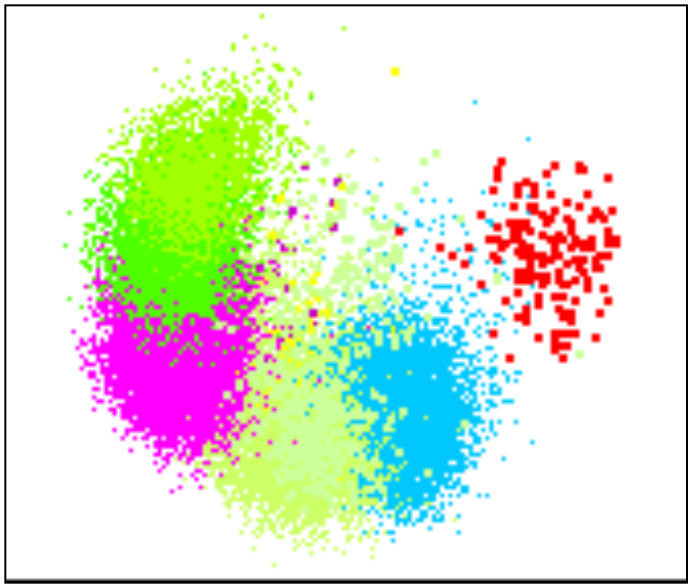
NAIVE CELLS

IGM+IGD+
MEMORY CELLS

IGM-IGD-
MEMORY CELLS

PLASMABLAST CELLS

Principal component analysis



Fast, linear

Hiding small

Projection of n-dimensions to 2D space

Principal component analysis

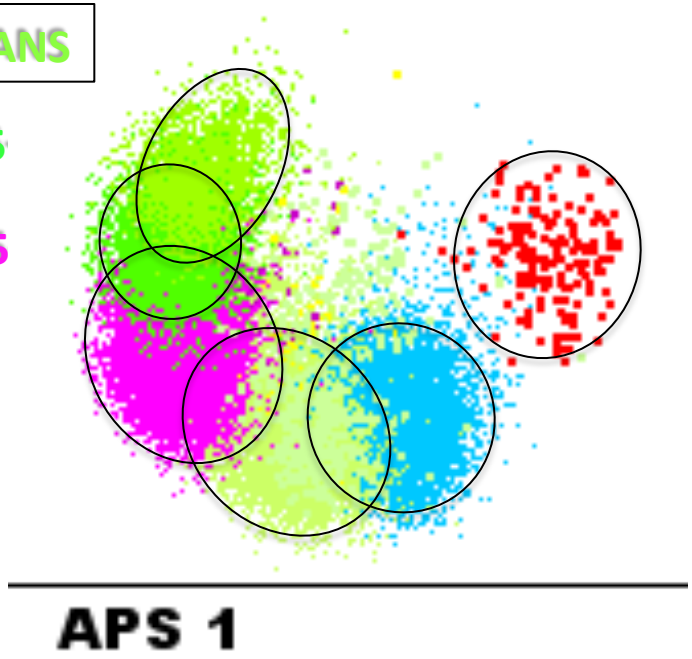
IMMATURE/TRANS

NAIVE CELLS CD5-

NAIVE CELLS CD5+

IGM+IGD+ MEMORY CELLS

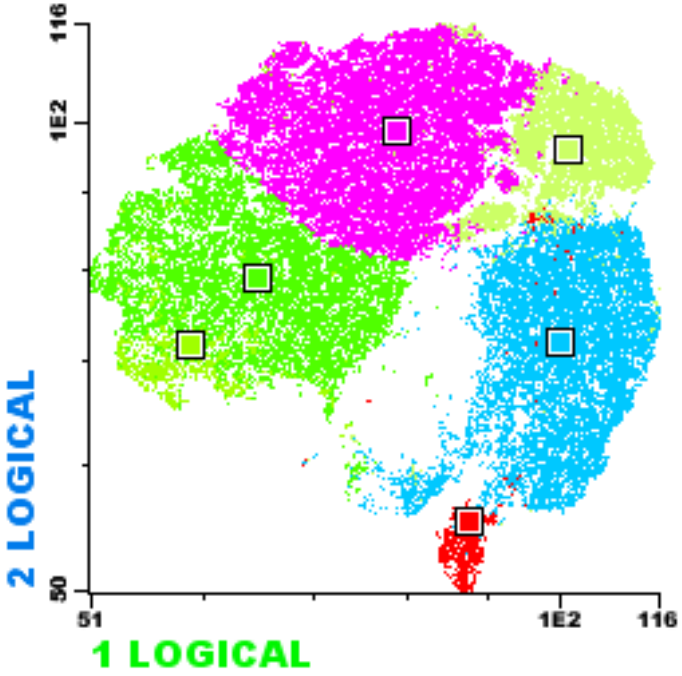
IGM-IGD- MEMORY CELLS



PLASMABLAST CELLS

Infinicyt software / R

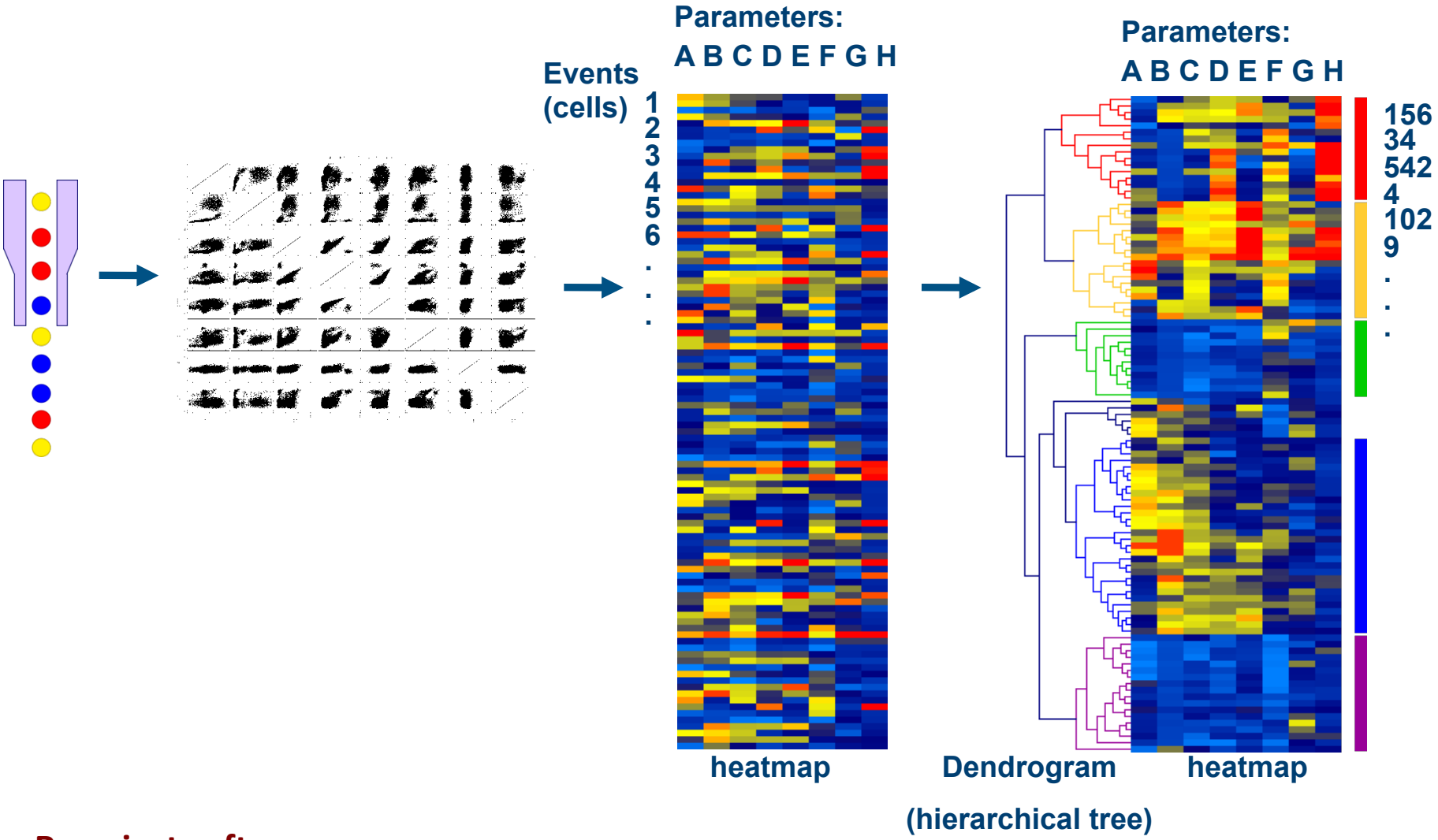
t-SNE 2Dmap



Representing all events

Slow, non-linear

Hierarchical Clustering Analysis
Re-order the data



R-project software

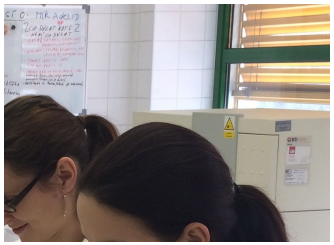
Fiser, ***Cytometry (2012)***

Data analysis - > Cytometry standardization

1. Sample prep
   Standard Operating Protocol (SOP)

2. Instrument setup SOP

3. Reagents
   fixed clone,



ESLHO european scientific foundation of laboratory hemato oncology — EuroFlow a division of ESLHO

EuroFlow Standard Operating Procedure

(SOP) for

sample preparation and staining

Version 1.2

23 September 2015

ESLHO european scientific foundation of laboratory hemato oncology — EuroFlow a division of ESLHO

Updated in Version 1.2

- Extension of incubation time for surface membrane staining
- Addition of general remark
- Adjustment in washing procedure (see Section 2.2 – 2.4)
- Adjustment in volume (See 2.14 page 4)
- Several textual adaptations have been introduced to improve clarity and avoid misinterpretation

EuroFlow Standard Operating Protocol (SOP) for

Instrument Setup and Compensation

EuroFlow a division of ESLHO

EuroFlow panel marker list:
Reference antibodies and alternative antibodies

Version 1.7 (11 February 2016)(updates in blue boxes outlined in red)

Disclaimer on EuroFlow reference reagents and equivalent reagents

EuroFlow reference reagents, obtained from multiple different companies, have been tested for usage at specific concentrations in the EuroFlow antibody combinations as reported in the EuroFlow documents. The recommended reagents aim to have comparable results in the (inter-laboratory) standardized EuroFlow staining procedures. However, EuroFlow cannot be responsible for any change in the manufacturing of the reagents, such as concentration, purity, dye/protein ratio, or any other factor that may affect the performance of these products both as single reagents and in the combinations in which they are included.

The so-called "equivalent reagents" have been tested as single reagents in comparison with the EuroFlow reference reagents. Performance of equivalent reagents in each of the individual EuroFlow 8-color combinations or modifications in the performance characteristics of equivalent reagents (e.g. in different lots) is out of the EuroFlow control.

Page 1 of 7

EuroFlow a division of ESLHO

marker list – Version 1.7 – **11 February 2016**

Reference/ Alternative	*Fluorochrome*	*Clone*	*Source*	*Catalogue number*	*Application in EuroFlow panel*
Reference	APC	H149	BD Biosciences	559775	T-ALL
Reference	FITC	RPA-2.10	BD Biosciences	555326	T-ALL
Reference	PacB	TS1/8	BioLegend	309216	NK-CLPD
Alternative	*HV450*	*IS-2*	*BD Biosciences*	*644485*	*See Footnote**
Reference	PECy7	S5.2	BD Biosciences	335821	T-CLPD
Reference	PacB	UCHT1	BD Biosciences	558117	ALOT, T-ALL
Alternative	*HV450*	*UCHT1*	*BD Biosciences*	*560365*	*See Footnote**
Alternative	*PacB*	*UCHT-1*	*Dako*	*P0982*	*See Footnote**
Reference	APC	SK7	BD Biosciences	345767	LST, SST, B-CLPD
Reference	APCH7	SK7	BD Biosciences	641415	ALOT, T-ALL
Reference	PerCPCy5.5	SK7	BD Biosciences	332771	T-CLPD, NK-CLPD
Reference	APCH7	SK3	BD Biosciences	641398	AML
Reference	PacB	RPA-T4	BioLegend	300521	LST, B-CLPD, T-CLPD
Alternative	*HV450*	*RPA-T4*	*BD Biosciences*	*560345*	*See Footnote**
Alternative	*HV450*	*SK3*	*BD Biosciences*	*651849*	*See Footnote**
Reference	PerCPCy5.5	SK3	BD Biosciences	332772	T-ALL
Reference	PerCPCy5.5	SK3	Cytognos	CYT-SLPC4-50 part of SST mixture	SST
Reference	APC	L17F12	BD Biosciences	345783	NK-CLPD
Reference	FITC	L17F12	BD Biosciences	345781	T-CLPD
Reference	PerCPCy5.5	L17F12	BD Biosciences	341109	LST, T-ALL, B-CLPD
Reference	APC	124-1D1	eBioscience	17-0079-42	ALOT, T-ALL, AML
Alternative	*APC*	*M-T701*	*BD Biosciences*	*653311*	*See Footnote**
Reference	FITC	4H9	BD Biosciences	347483	T-CLPD, NK-CLPD
Reference	APCH7	SK1	BD Biosciences	641400	T-CLPD
Reference	FITC	UCH-T4	Cytognos	CYT-SLPC-50 part of LST mixture	LST, B-CLPD
Reference	FITC	UCH-T4	Cytognos	CYT-SLPC4-50 part of SST mixture	SST
Alternative	*FITC*	*SK1*	*BD Biosciences*	*345772*	*See Footnote**
Alternative	*FITC*	*DK25*	*Dako*	*F0765*	*See Footnote**
Reference	PECy7	SFCI21Thy2D 3	Beckman Coulter	737661	T-ALL
Reference	APCH7	M-L13	BD Biosciences	655409	AML
Reference	PacB	MEM-61	Exbio	PB-208-T100	BCP-ALL
Alternative	*HV450*	*M-L13*	*BD Biosciences*	*658167*	*See Footnote**
Reference	APC	H10A	BD Biosciences	332777	BCP-ALL

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Leukemia (2012) **26**, 1908–1975
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www.nature.com/leu

Open

ORIGINAL ARTICLE

EuroFlow antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes

JJM van Dongen[1], L Lhermitte[1], S Böttcher[2], J Almeida[3], VHJ van der Velden[1], J Flores-Montero[4], A Rawstron[5], V Asnafi[6], Q Lécrevisse[3], P Lucio[4], E Mejstrikova[7], T Szczepański[8], T Kalina[7], R de Tute[9], M Brüggemann[2], L Sedek[8], M Cullen[9], AW Langerak[1], A Mendonça[10], E Macintyre[6], M Martin Ayuso[3], O Hrusak[7], MB Vidriales[11] and A Orfao[3] on behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

Most consensus leukemia & lymphoma antibody panels consist of lists of markers based on expert opinions, but they have not been validated. Here we present the validated EuroFlow 8-color antibody panels for immunophenotyping of hematological malignancies. The single-tube screening panels and multi-tube classification panels fit into the EuroFlow diagnostic algorithm with entries defined by clinical and laboratory parameters. The panels were constructed in 2–7 sequential design–evaluation–redesign rounds, using novel InForm1 software tools for multivariate data analysis. Two groups of markers are combined in each 8-color tube: 3) backbone markers to identify distinct cell populations in a sample, and iii) markers for characterization of specific cell populations. In multi-tube panels, the backbone markers were optimally placed at the same fluorochrome position in every tube, to provide identical multidimensional localization of the target cell population(s). The characterization markers were positioned according to the diagnostic utility of the combined markers. Each proposed antibody combination was tested against reference databases of normal and malignant cells from healthy subjects and WHO-based disease entities, respectively. The EuroFlow studies resulted in validated and flexible 8-color antibody panels for multidimensional identification and characterization of normal and aberrant cells, optimally suited for immunophenotypic screening and classification of hematological malignancies.

Leukemia (2012) **26**, 1908–1975; doi:10.1038/leu.2012.120

Keywords: EuroFlow; antibody panel; lymphoma; flow cytometry; 8-color immunostaining; standardization; hematological malignancies

INTRODUCTION

For more than two decades, immunophenotyping has been providing relevant information for the diagnosis, classification and monitoring of hematological malignancies.[1–7] Together with cyto/histomorphology and molecular cytogenetics, immunophenotyping is crucial for the identification, enumeration and characterization of leukemia and lymphoma cells. Consequently, it has acquired a prominent position in the current World Health Organization (WHO) classification of hematological malignancies.[8] Preferably, the immunophenotypic profiles of suspected cells should be compared with those of normal hematopoietic cells. Immunophenotypic similarities between the suspected cells and their potential normal counterparts allow the assignment of such cells to a given hematopoietic cell lineage and maturational stage, as well as the identification of aberrant phenotypes, such as leukemia-associated immunophenotypes.[9–11] Such immunophenotyping requires careful selection of unique combinations of individual markers based on their degree of specificity for the identification of a given cell lineage, maturation stage and aberrant phenotype, as well as the selection of appropriate antibody clones and fluorochrome conjugates to be used in multicolor combinations; the performance of these marker combinations is even more relevant than that of the individual markers. Consequently, such careful selection of reagents is essential for the design of standardized multicolor antibody combinations that provide unique staining patterns for each normal or aberrant cell population in a given sample.

Each marker combination should be designed to answer one or multiple relevant clinical questions, through the identification, enumeration and characterization of the relevant cell populations in a sample. As the target cell population may not be known in advance or might have been defined previously, a different strategy is required in each situation. In the former situation, a rapid screening step based on a limited number of antibodies (preferably in a single tube) directed at differential identification of all relevant cell subsets in the sample is generally most

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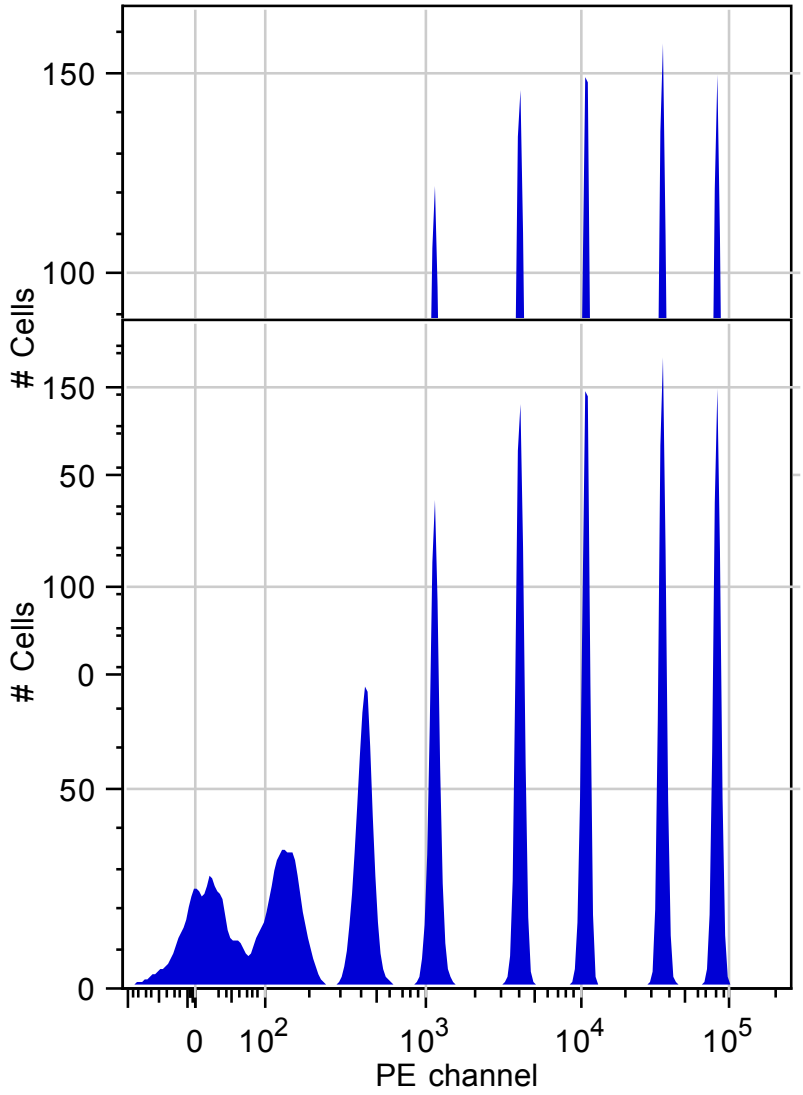
Received 27 March 2012; revised 14 February 2012; accepted 19 April 2012; accepted article preview online 3 May 2012

.euroflow.org
Montero at jflores@usal.es

van Dongen et al., **Leukemia**, 2012
Kalina et al., **Leukemia**, 2012
www.euroflow.org

Signal position x PMT voltage

We aim for a signal at the same position

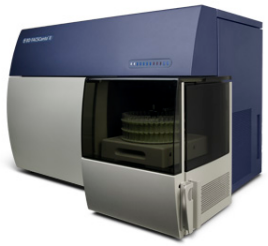


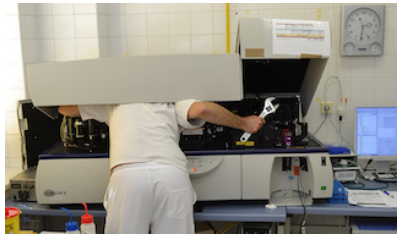
- Beads are ideal for that (Rainbow, CST)

(Rainbow are not spectrally matched)

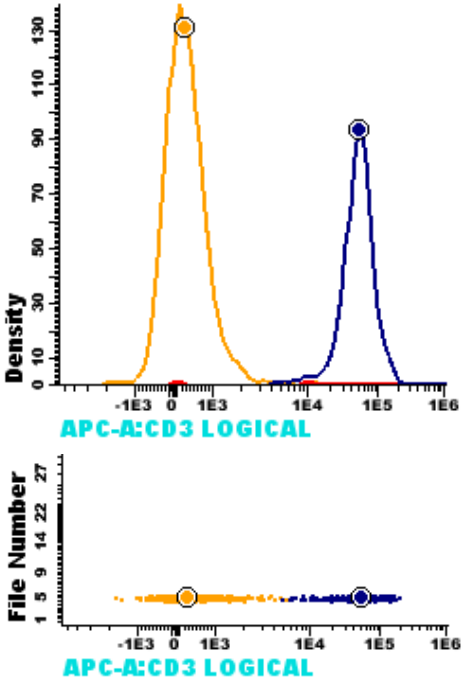
Standardization – what is it?

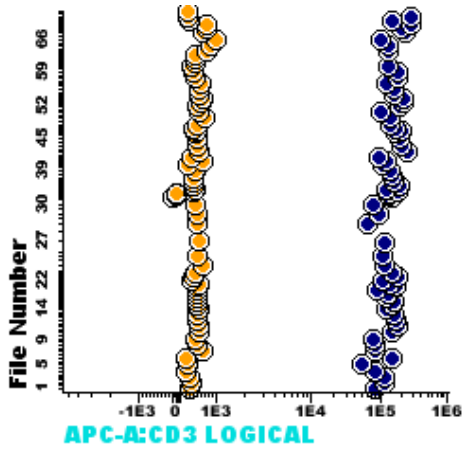
4. Data acquisition



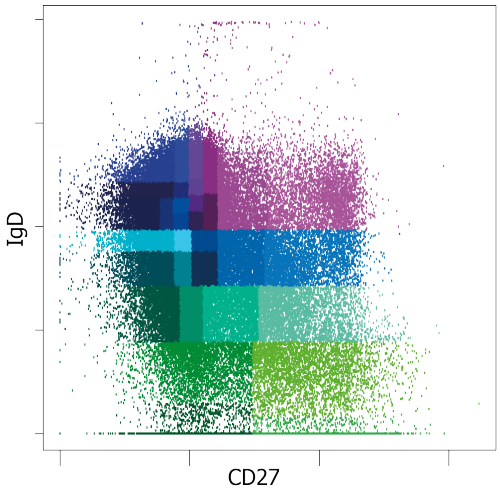


5. Data analysis

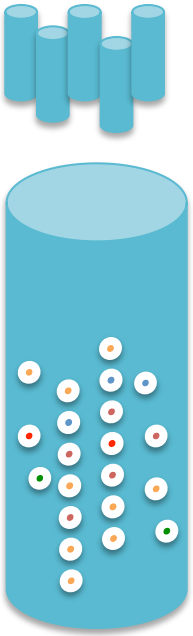




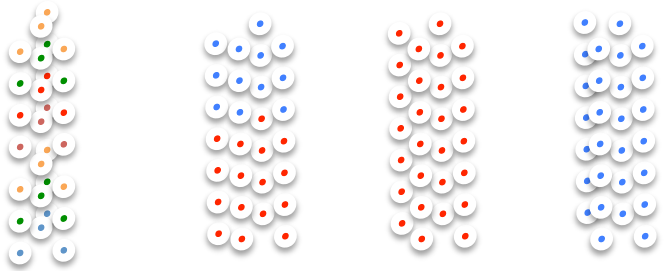
Polychromatic space analysis
Cohort analysis



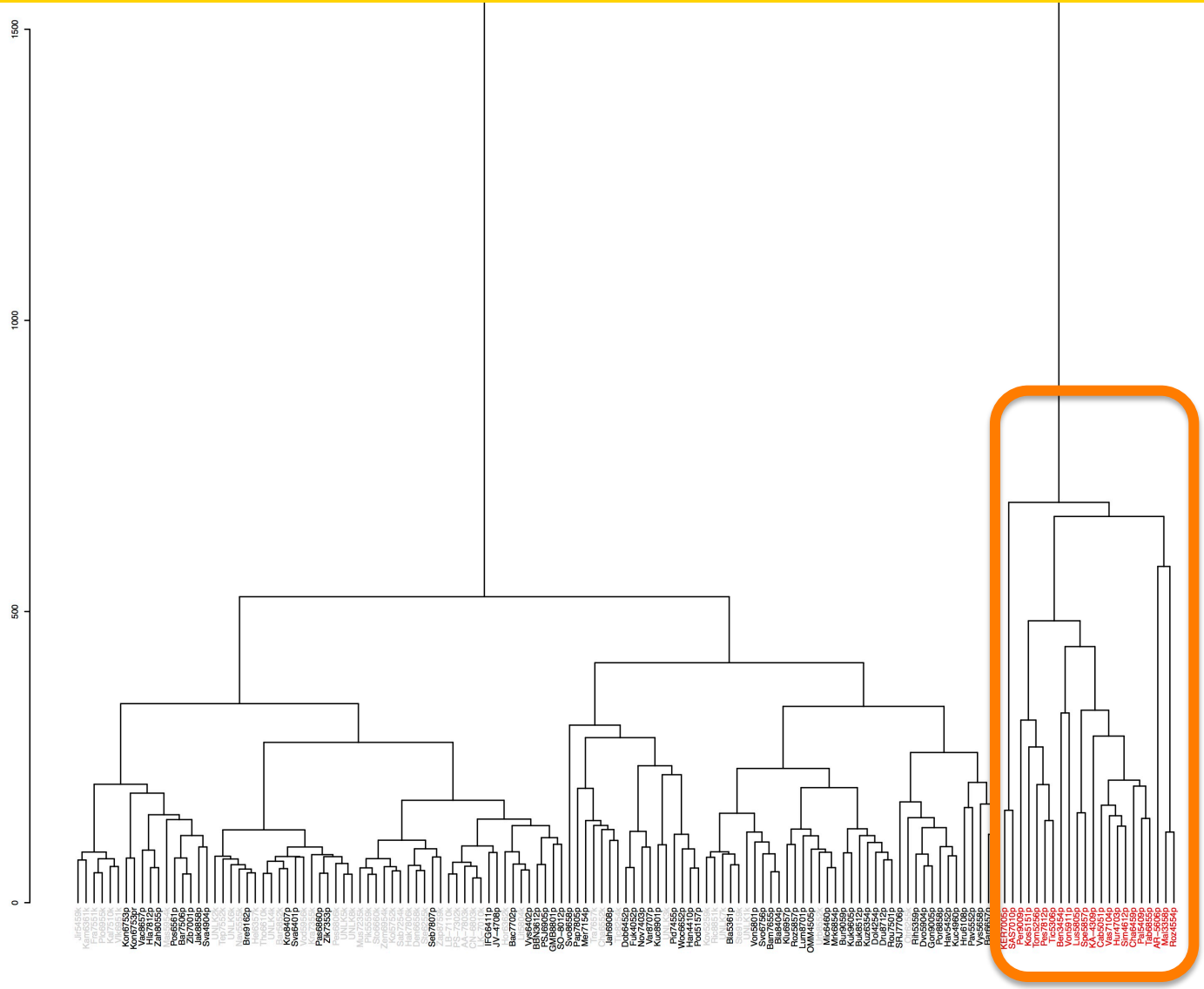
Kalina, **Cytometry A**, **2009**.
Stuchlý**, Sci Rep, 2017**



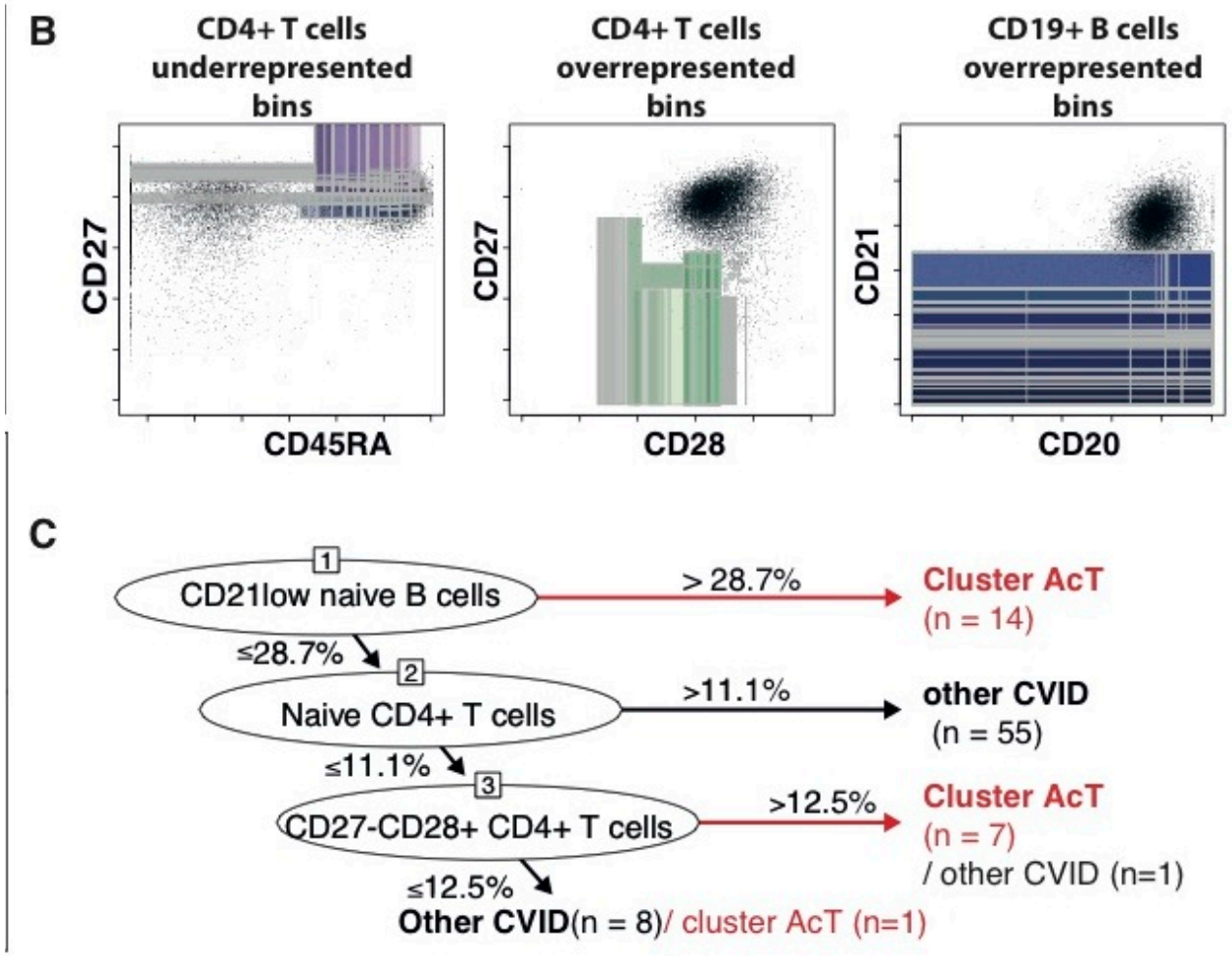
8-color B-cell profile
98 CVID patients and 47 healthy controls



Cluster of patients according to the B and T cell profile

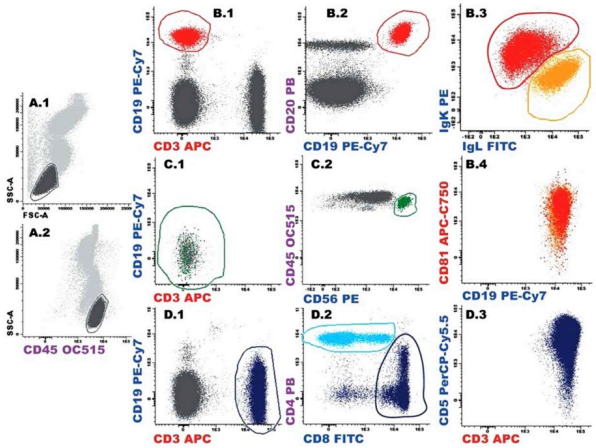


What are the major features of the cluster in known gates?

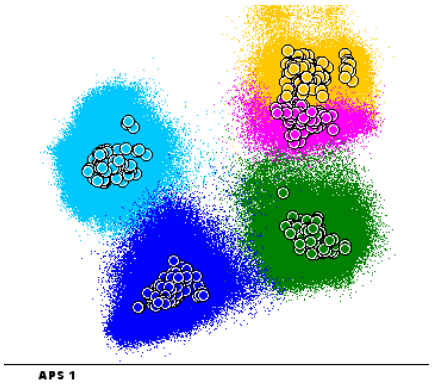


Data analysis tools for cohorts

Common gating

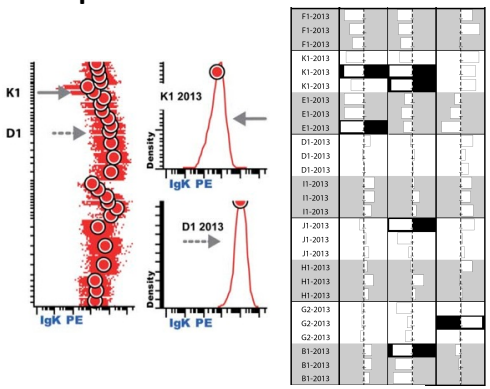


Cohort - multivariate



Layout + Batch analysis

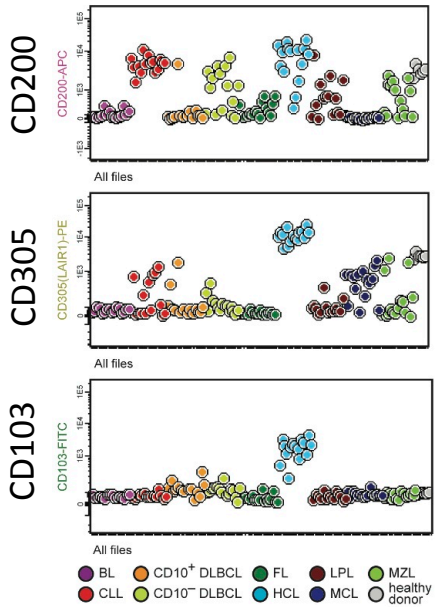
QA - comparison to expected fluorescence



$$p - score = \frac{\log_{10} MedFI - \log_{10} qaMedFI}{D^{\max}}$$

Kalina, **Cytometry A**, 2015

Cohort - univariate



FCS file number ->

Algorithmic analysis

Software - overview

- FlowJo (Mac & Win) – batch gating, statistics/tables, overlays, stacked histograms, t-SNE (PCA)
- Diva – layouts/batch
- Kaluza – Radar plot, Tree plots, layouts ?
- Infinicyt – PCA, databases - automated analysis
- Cytobank – ViSNE, SPADE, histograms
- Summit
- R-project

- FCS Express
- Flowing software
- Cyflogic
- Cytospec and PlateAnalyzer

Hardware

Dataserver (few Tb)

PC (as for games, no audio)
- SSD disk (prevent overfilling)
- RAM (16GB or more)
- Fast graphics
- Good monitor

Tip: Software and hardware is expensive, add it to instrument purchase

Sharing the knowledge

- Quality description of experiments

ORIGINAL ARTICLE

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

MIFlowCyt: The Minimum Information About a Flow Cytometry Experiment

Jamie A. Lee,[1†] Josef Spidlen,[2†] Keith Boyce,[3] Jennifer Cai,[1] Nicholas Crosbie,[4] Mark Dalphin,[5] Jeff Furlong,[6] Maura Gasparetto,[2] Michael Goldberg,[7] Elizabeth M. Goralczyk,[8] Bill Hyun,[9] Kirstin Jansen,[6] Tobias Kollmann,[10] Megan Kong,[1] Robert Leif,[11] Shannon McWeeney,[12,13,14] Thomas D. Moloshok,[8] Wayne Moore,[15] Garry Nolan,[16] John Nolan,[17] Janko Nikolich-Zugich,[18] David Parrish,[3] Barclay Purcell,[19] Yu Qian,[1] Biruntha Selvaraj,[19] Clayton Smith,[2] Olga Tchuvatkina,[7] Anne Wertheimer,[20] Peter Wilkinson,[21] Christopher Wilson,[6] James Wood,[22] Robert Zigon,[23] The International Society for Advancement of Cytometry Data Standards Task Force, Richard H. Scheuermann,[1,24] Ryan R. Brinkman[2*]

- FCS data availability

https://flowrepository.org/

Basic Multicolor Flow Cytometry

Fluorochromes, Spillover and Compensation

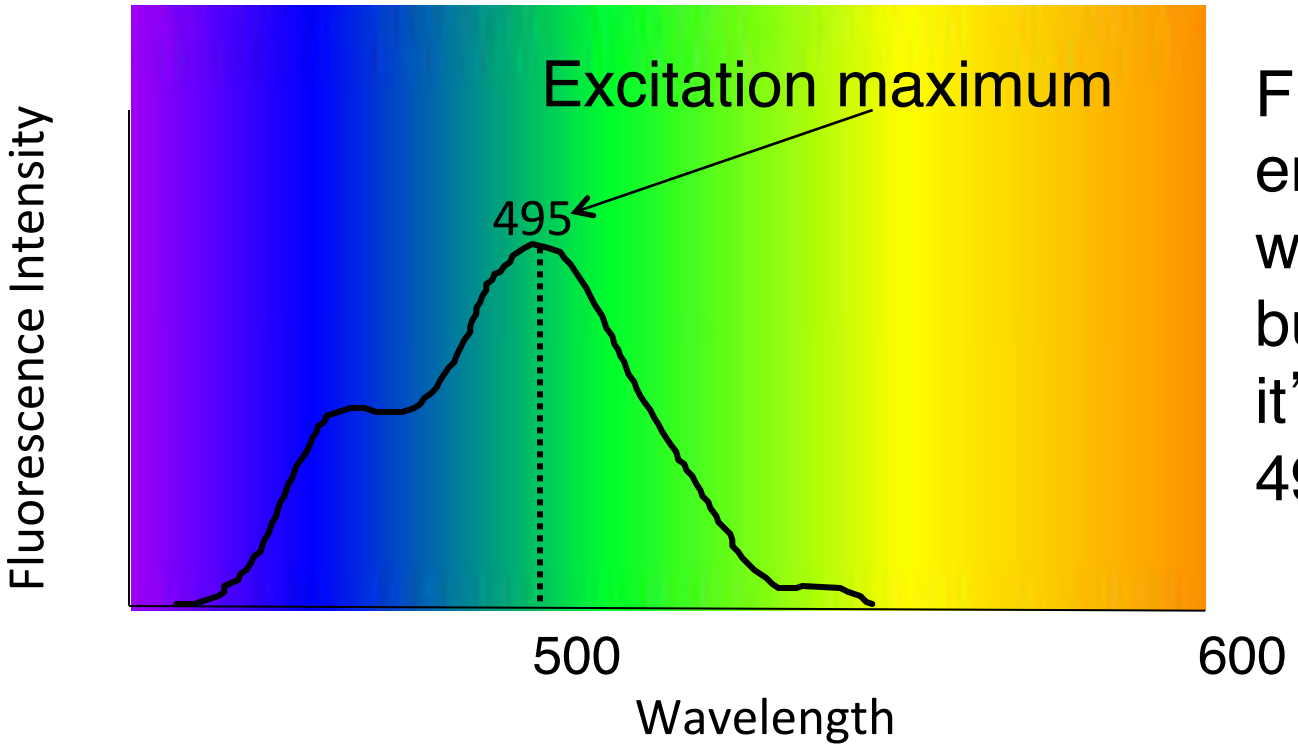
Zosia Maciorowski

Insitut Curie

Paris, France

Excitation spectrum

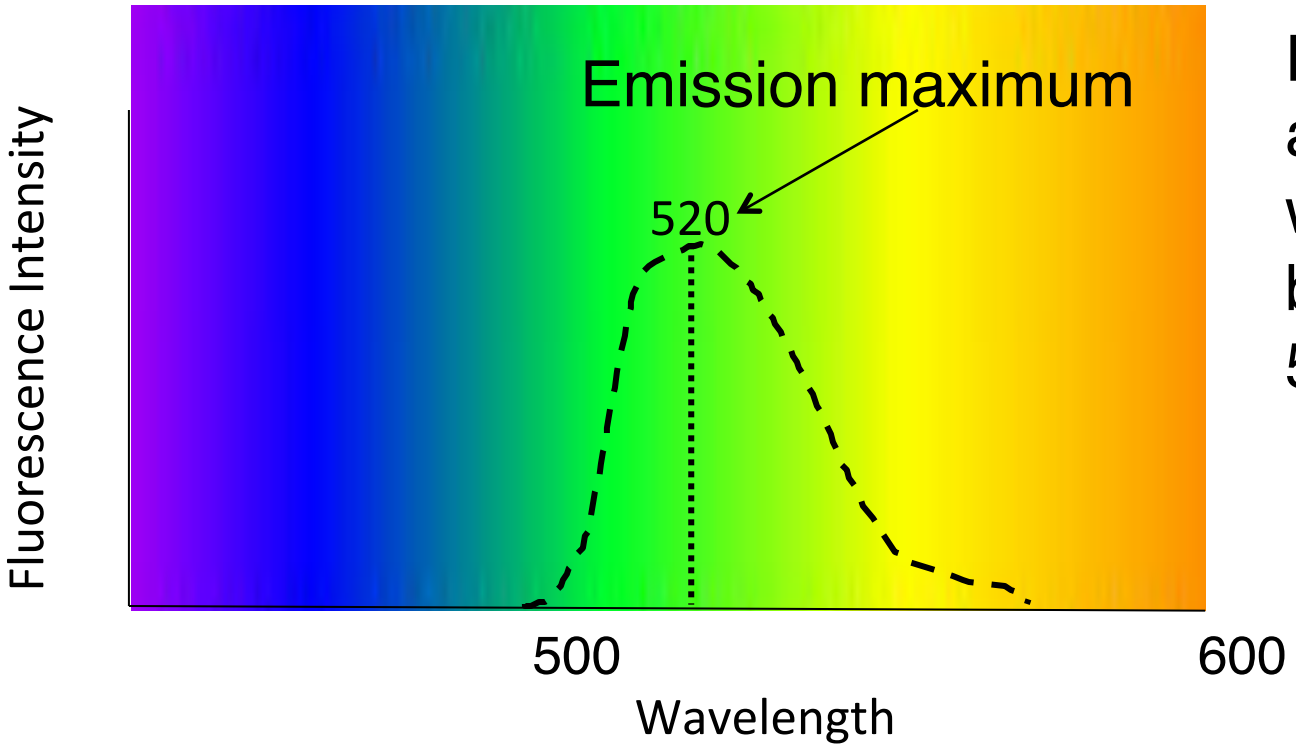
Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths



FITC can absorb energy at all these wavelengths but absorbs best at it's excitation max: 495nm

Emission spectra

Each fluorochrome is also capable of emitting light energy over a specific range of wavelengths

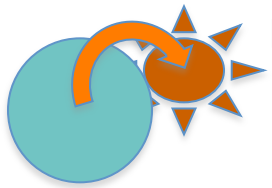


FITC will fluoresce at all these wavelengths but highest at 520nm

Tandem Dyes have 2 fluorochromes coupled together

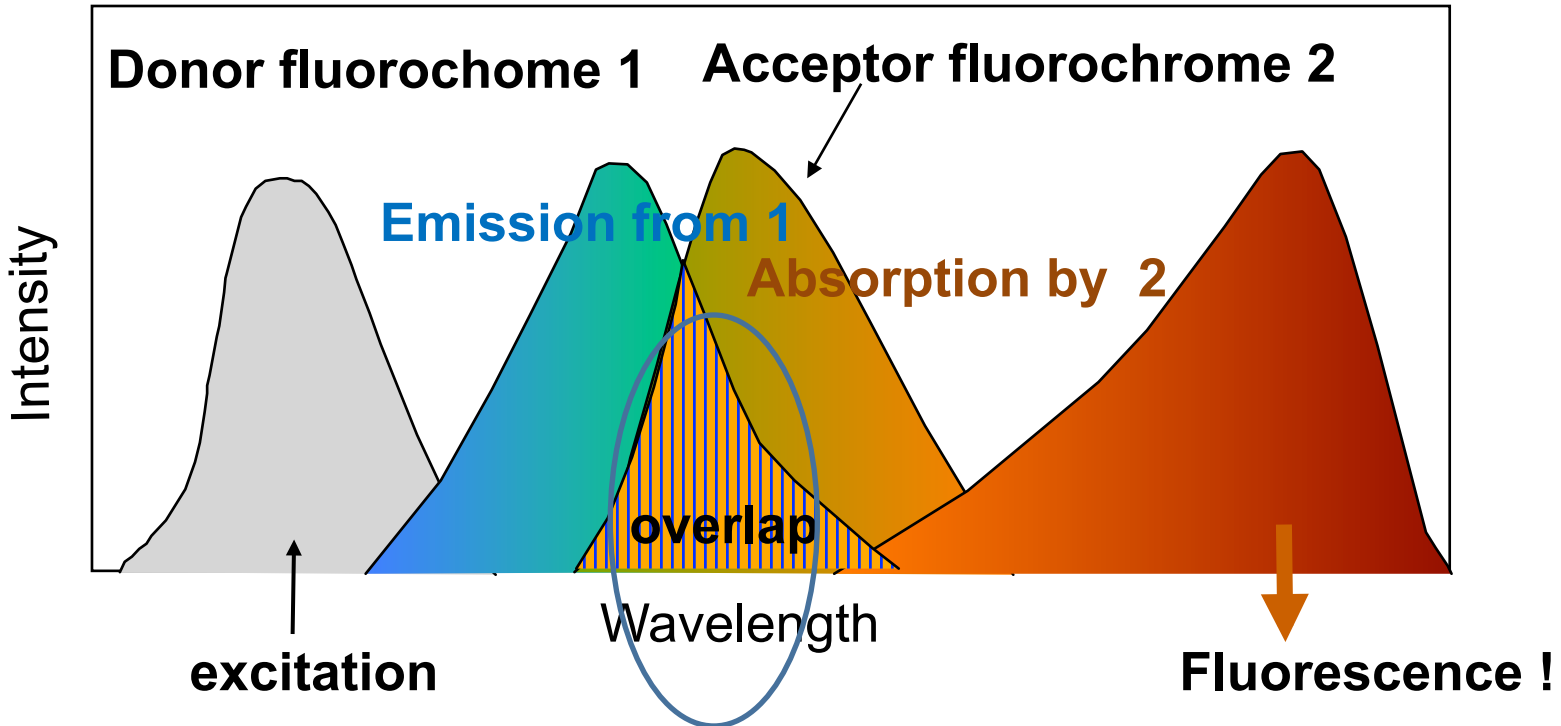
Energy Transfer

The 1st fluorochrome transfers it's absorbed energy to the 2nd fluorochrome



Fluorochrome 2

Fluorochrome 1



Tandem Dyes: conditions

Energy transfer:

- Effective between 10-100 Å only
- Emission and excitation spectrum must significantly overlap
- Donor transfers non-radiatively to the acceptor

Tandem dyes: caution

- all tandems are not the same.

- Some batches of tandems have better coupling and therefore better energy transfer than other batches.

- This means there is more or less leakage from the first fluorochrome

- More or less compensation will be necessary in that emission channel.

- ALWAYS use the same tandem in your single colors as you use in your mix!!

Commonly used tandems

PE-Cy5

PerCP-Cy5.5

PE-Texas Red

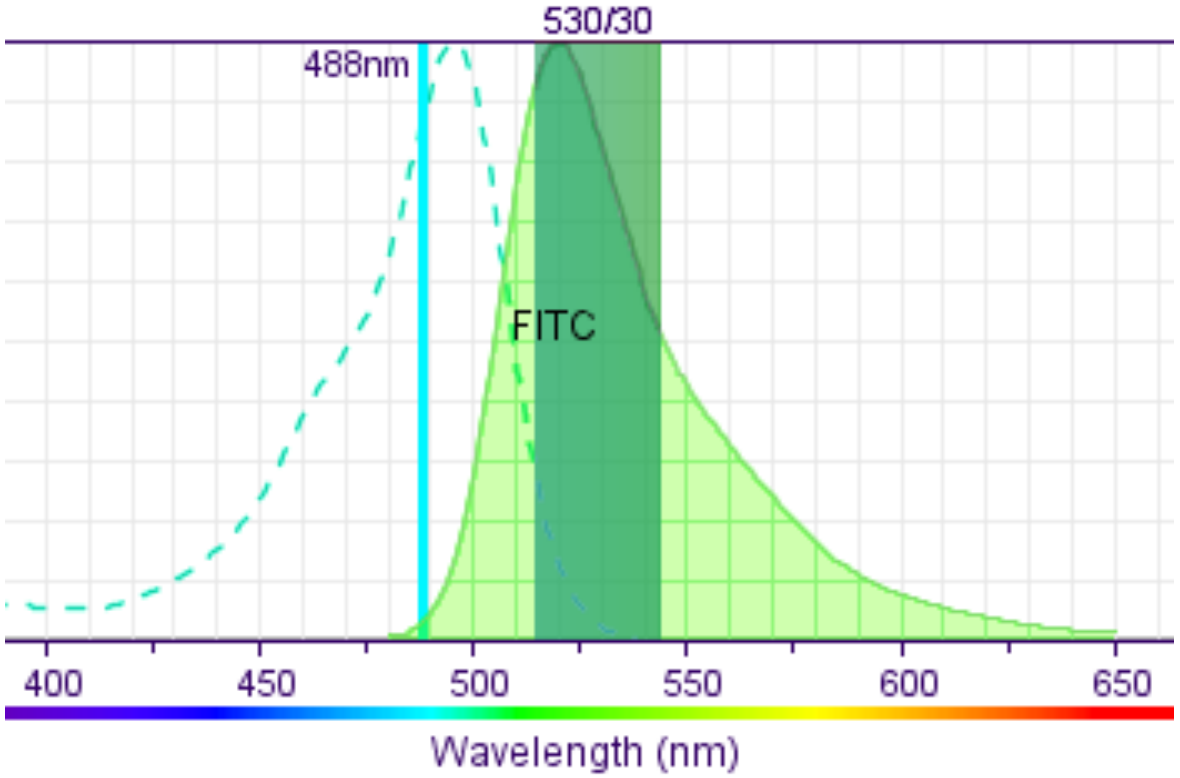
Pe-Cy7

APC-Cy7

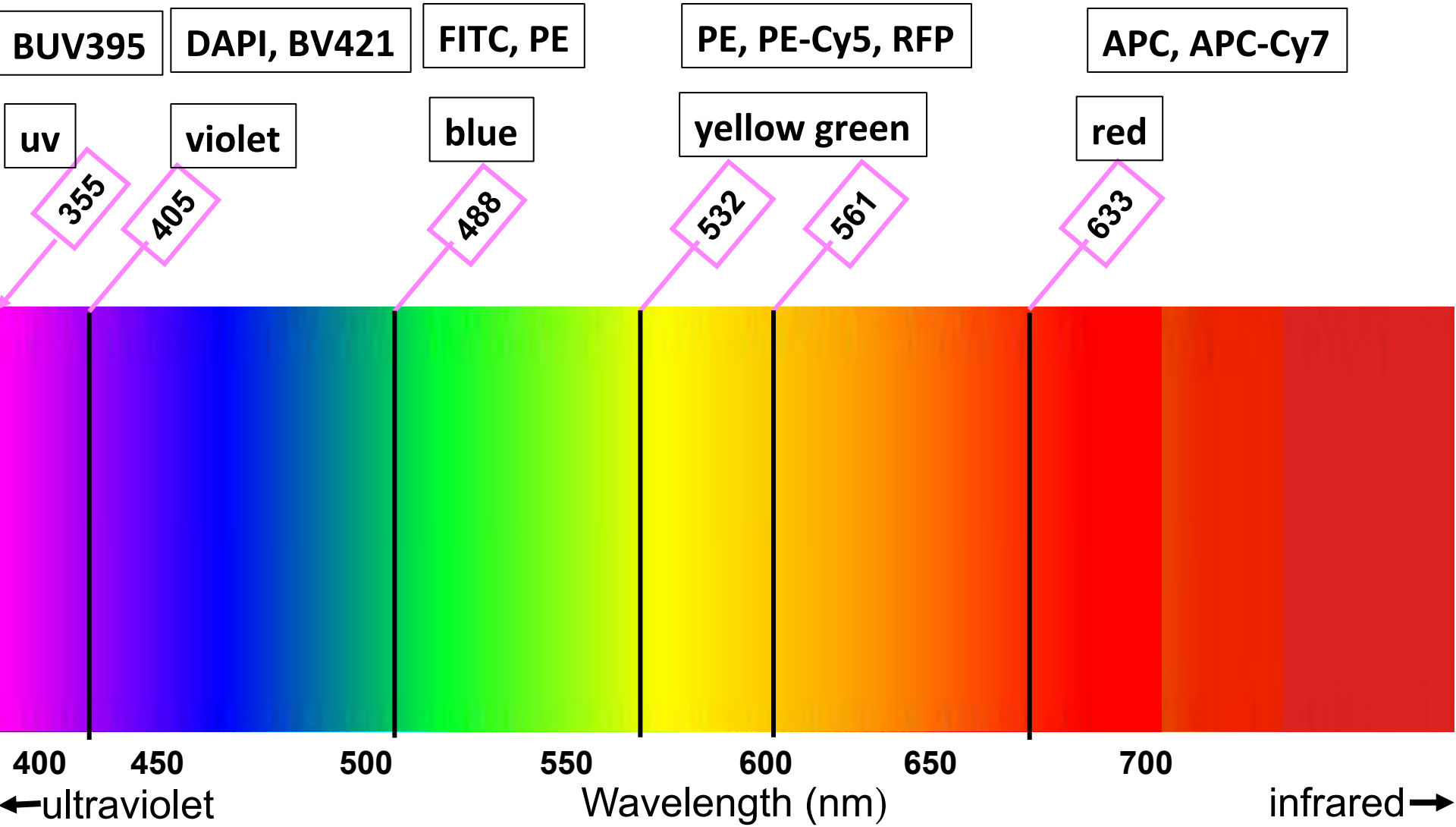
Brilliant (Sirigen) dyes (Brilliant Violet)

Choosing Fluorochromes: which lasers and filters?

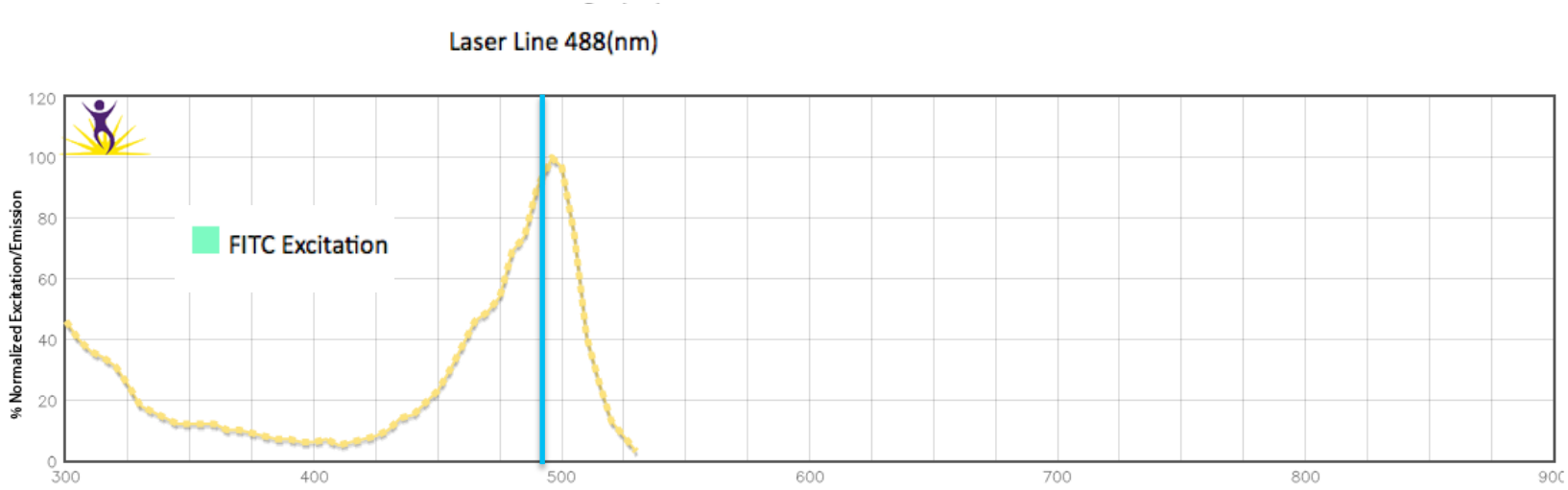
- Look at the **excitation** spectra to determine which lasers can be used to excite the fluorochrome.
- Look at the **emission** spectra to determine which filters should be used to collect the signal.



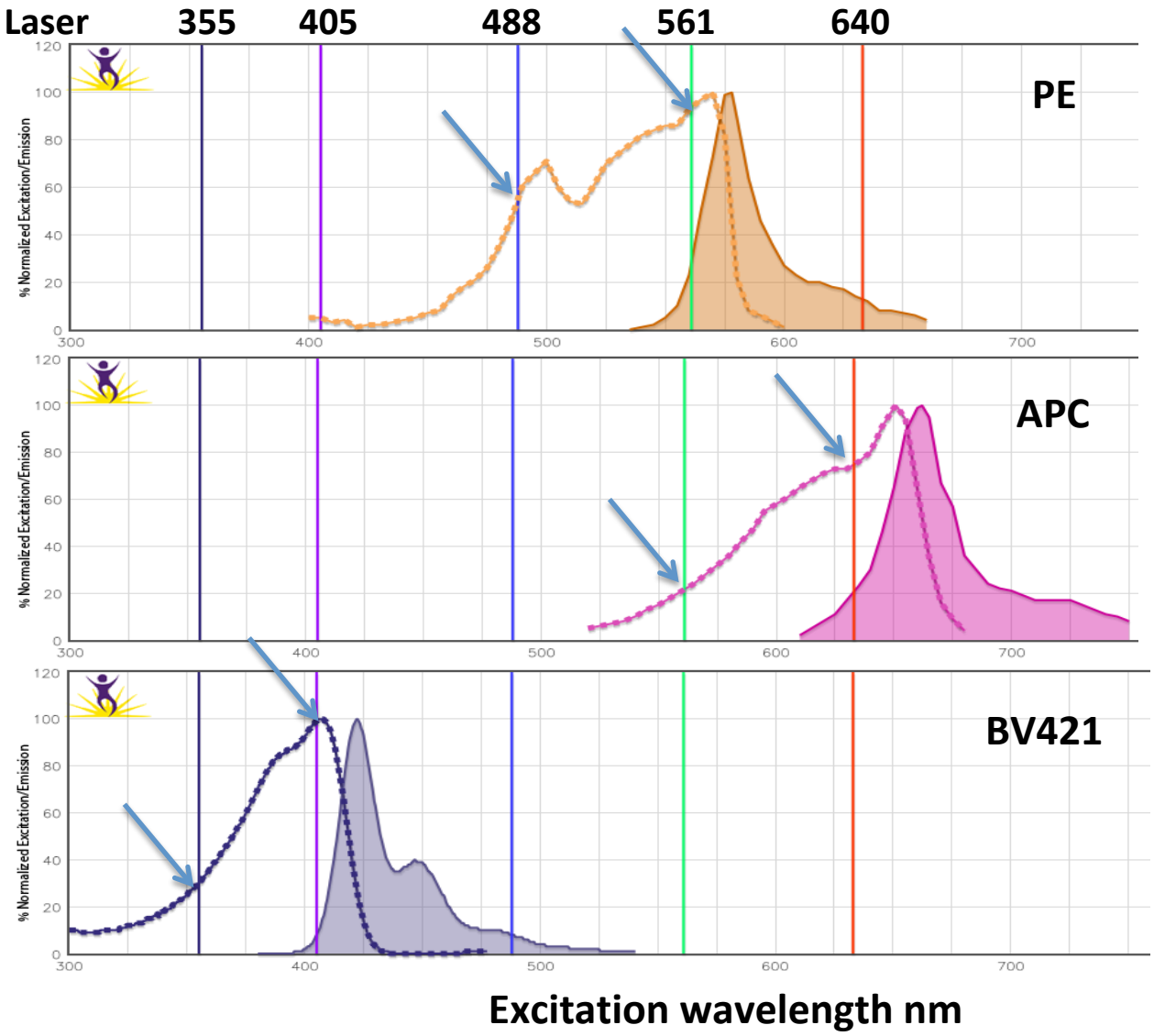
Laser wavelengths on typical cytometers



Look at the excitation spectra of your fluorochromes



Fluorochromes can be excited by different lasers.



PE has a wide excitation spectrum, excited by the 488 laser, but more efficiently with the 561

APC is excited by the 640 but also a little by the 561

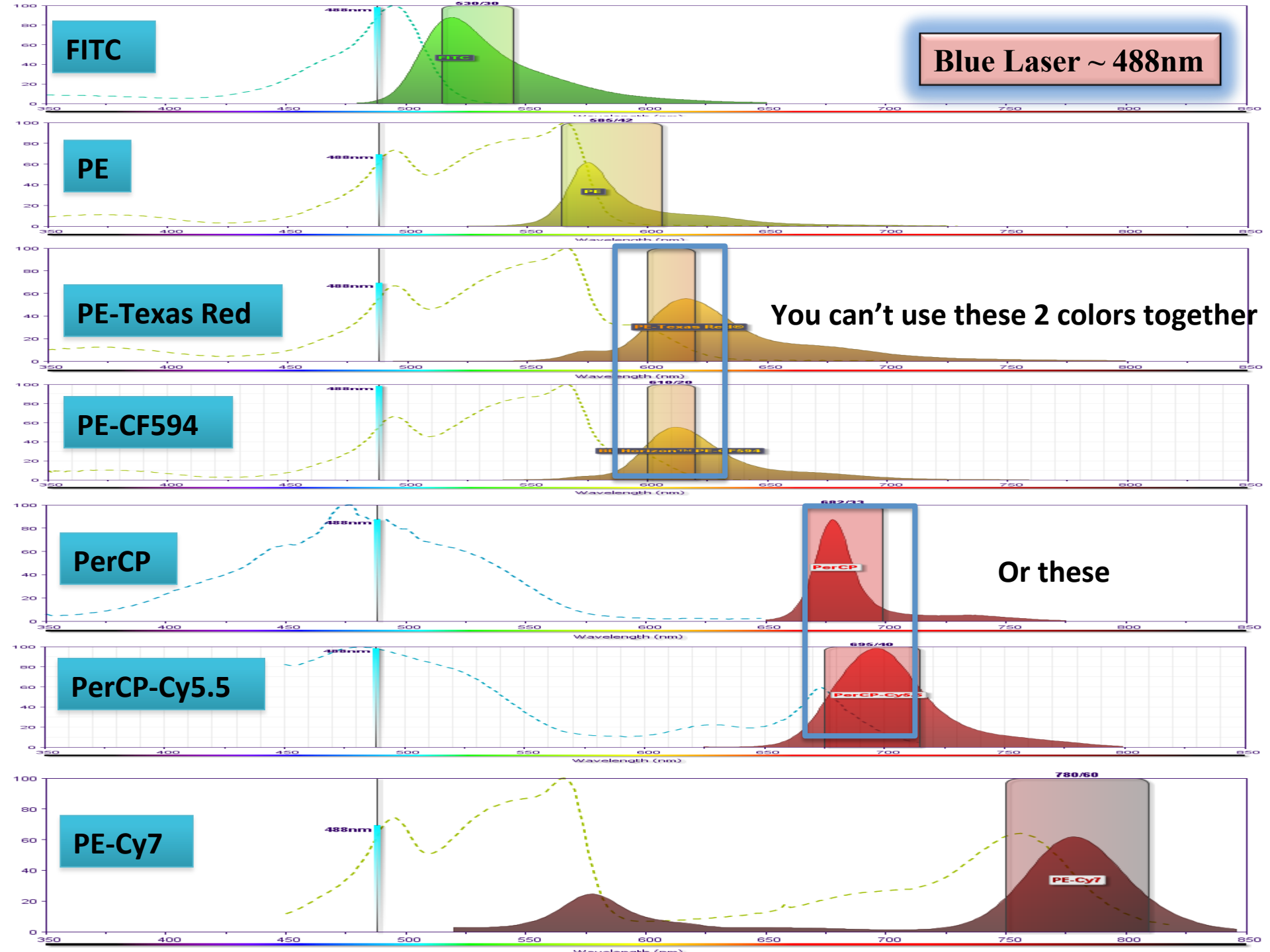
BV421 is excited best by the 405 laser but also a little by the 355.

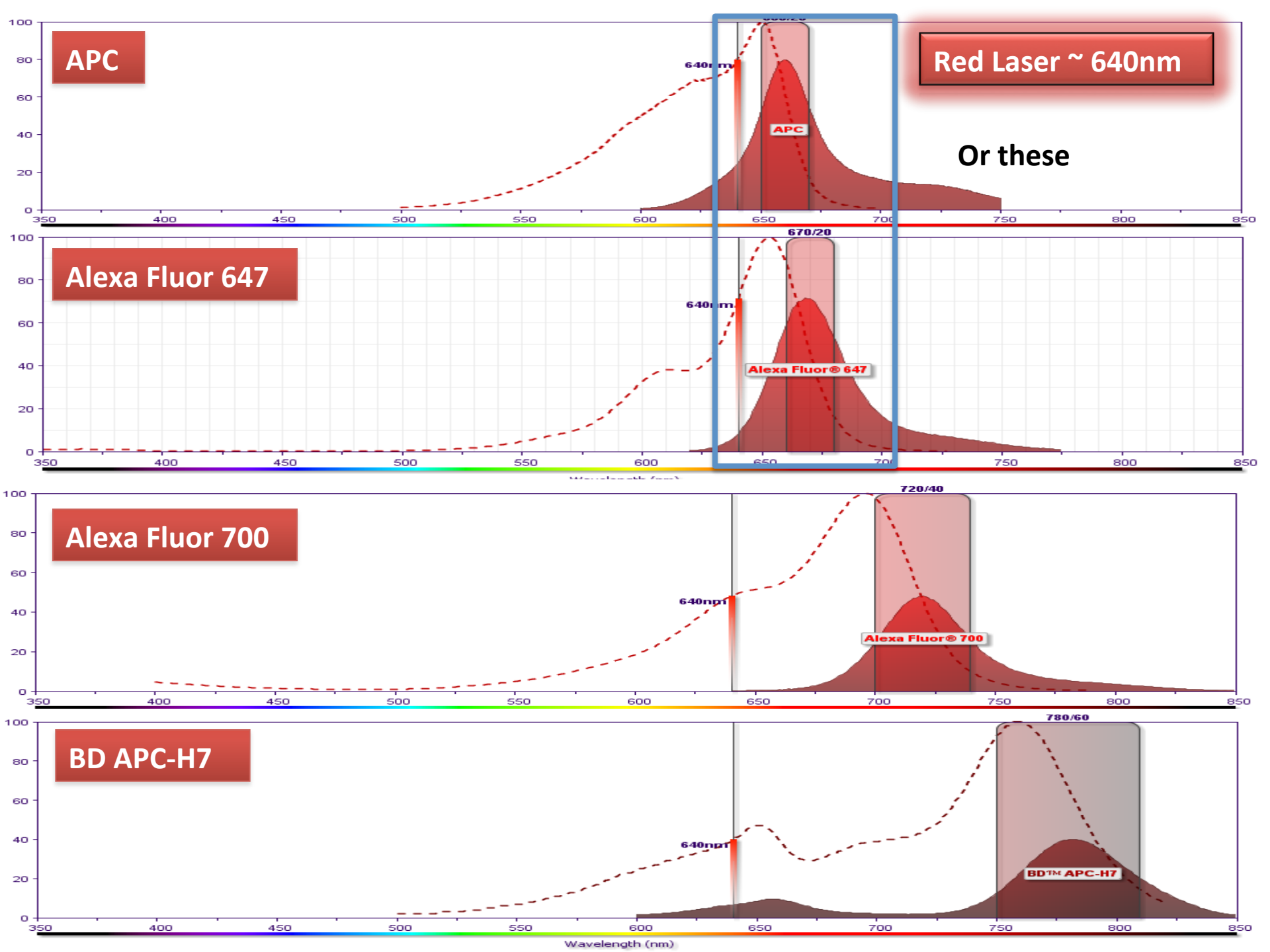
Emission spectra

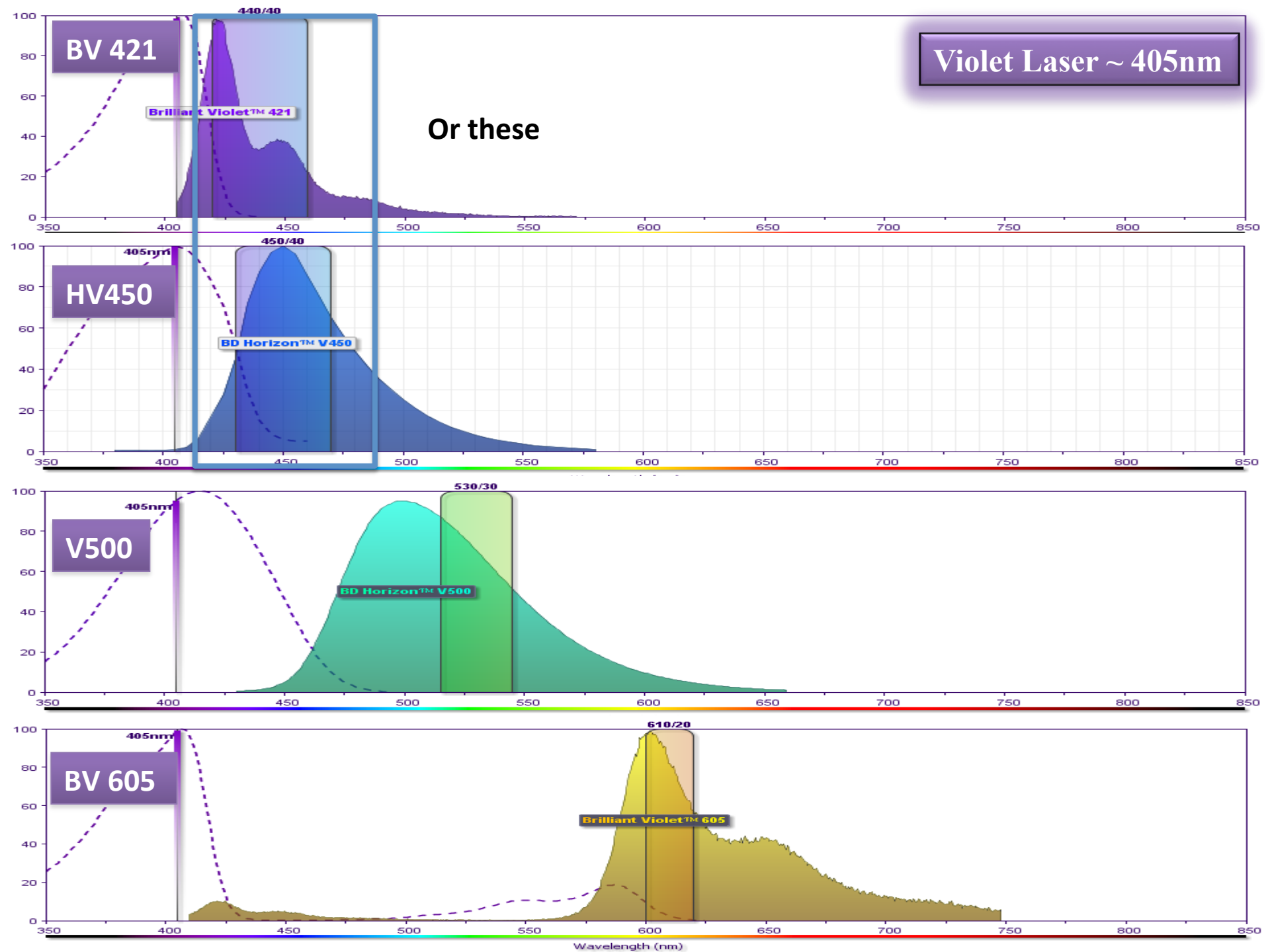
Look at the emission spectra of your fluorochromes

Make sure the filters on your cytometer correspond to the maximum emission

Fluorochromes excited off the same laser should not have overlapping emission spectra


FITC
Blue Laser ~ 488nm
PE
PE-Texas Red
You can't use these 2 colors together
PE-CF594
PerCP
Or these
PerCP-Cy5.5
PE-Cy7
488nm
Wavelength (nm)


APC
Red Laser ~ 640nm
Or these
APC
Alexa Fluor 647
670/20
Alexa Fluor® 647
Alexa Fluor 700
720/40
Alexa Fluor® 700
BD APC-H7
780/60
BD™ APC-H7
Wavelength (nm)


Violet Laser ~ 405nm
BV 421
440/40
Brilliant Violet™ 421
Or these
HV450
405nm
450/40
BD Horizon™ V450
V500
405nm
530/30
BD Horizon™ V500
BV 605
405nm
610/20
Brilliant Violet™ 605
Wavelength (nm)

Lots of “spectral viewers” online

- http://www.bdbiosciences.com/us/s/spectrumviewer
- http://www.biolegend.com/spectraanalyzer
- https://www.thermofisher.com/cn/zh/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

How bright is your fluorochrome?

Brightness is intrinsic to the fluorochrome itself

depends on: Extinction coefficient (light absorbance)

Quantum yield (photons out/photons in)

Fluorochrome	Extinction coefficient	Quantum yield	Brightness x 10^5	Brightness relative to PE	Size Daltons
PE	1,960,000	.84	16	100%	240,000
PeCy5	1,960,000	NA	NA	NA	241,500
APC	700,000	.68	4.7	29%	105,000
FITC	75,000	.5	0.4	2%	389
BV421	2,500,000	.69	16	100%	264

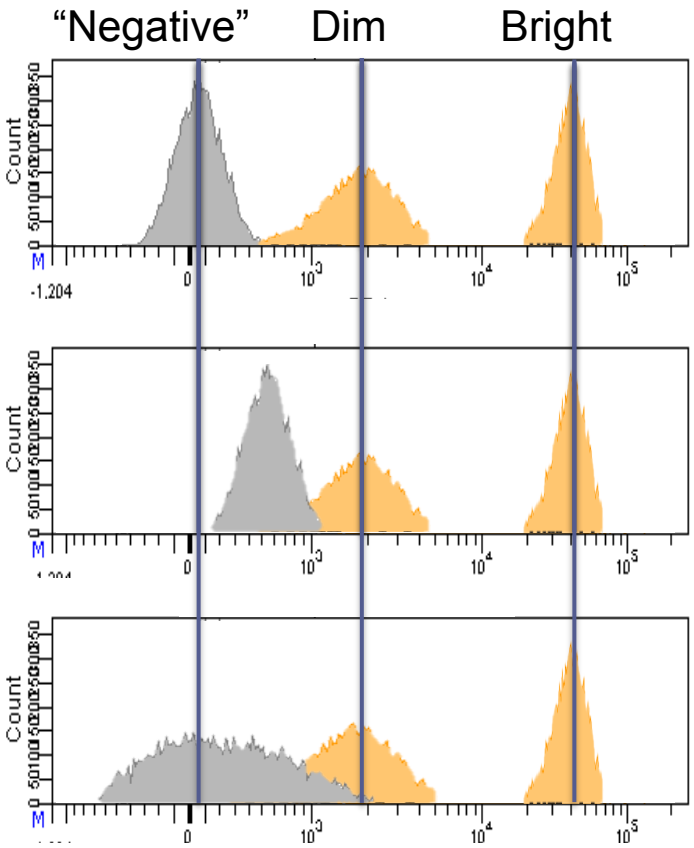
Resolution

We need to distinguish unstained from dimly stained in a mixture.

Negative population has low background
populations well resolved

Negative population has high background
Dim population not resolved

Negative population has low background but high spread (SD)
Dim population not resolved



The ability to resolve populations is a function of both background *and* spread of the negative population.

Q and B: criteria for cytometer performance

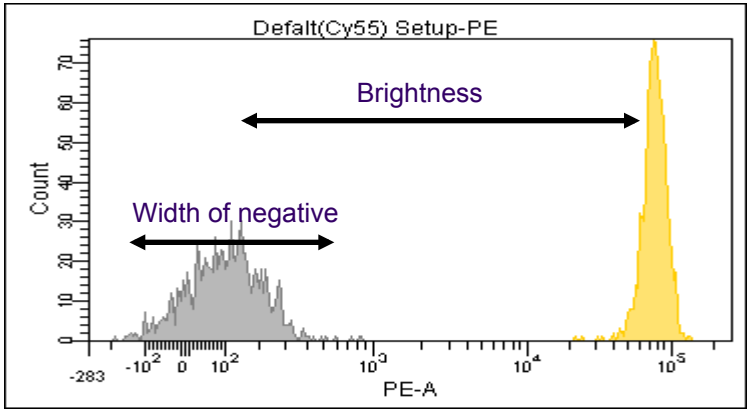
- Used to quantify detection sensitivity and background
- Q: number of photoelectrons produced per molecule of fluorochrome: sensitivity
- B: electronic and optical background when no fluorochrome is present
- Best detection when high Q and low B

Stain Index: How well separated are your populations

Stain index is a measure of reagent performance on a specific cytometer

Affected by fluorochrome brightness **AND instrument characteristics**

$$\text{Stain Index} = \frac{\text{median}_{\text{positive}} - \text{median}_{\text{negative}}}{2 \times \text{rSD}_{\text{negative}}}$$



Experiment Name: CD4 Stain Intex (081304)
Tube Name: PE

Population	#Events	%Parent	PE-A Median	PE-A rSD
PE-	827	48.9	102	97
PE+	780	46.1	75,652	12,616

$$\text{Stain Index} = \frac{75852 - 102}{2 \times 97} = 390.5$$

Stain Index Comparisons

Stain Index on Cytometer A

Reagent	CD4 Clone	Filter Set	Stain Index	Relative Brightness (compared to PE)
PE	RPA-T4	585/40	**305**	100.00%
PE-Cy5	RPA-T4	695/40	**198**	81.63%
PerCP	RPA-T4	695/40	**30**	16.66%
APC	RPA-T4	660/20	**263**	8.21%
FITC	RPA-T4	530/30	**43**	1.74%
Pacific Blue™	RPA-T4	440/40	**63**	1.5%

- **APC has a higher stain index than PE-Cy5 even though 1/10th as bright**
- **Due to less noise in red laser, thus background width is lower and stain index higher.**

Stain Index: instrument variation

Fluorochrome	Brightness x 10^5	Brightness relative to PE	Stain index	
			Cytometer 1	Cytometer 2
PE	16	100%	348	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	281
FITC	0.4	2%	132	61
BV421	16	100%	264	145

Due to cytometer differences in:

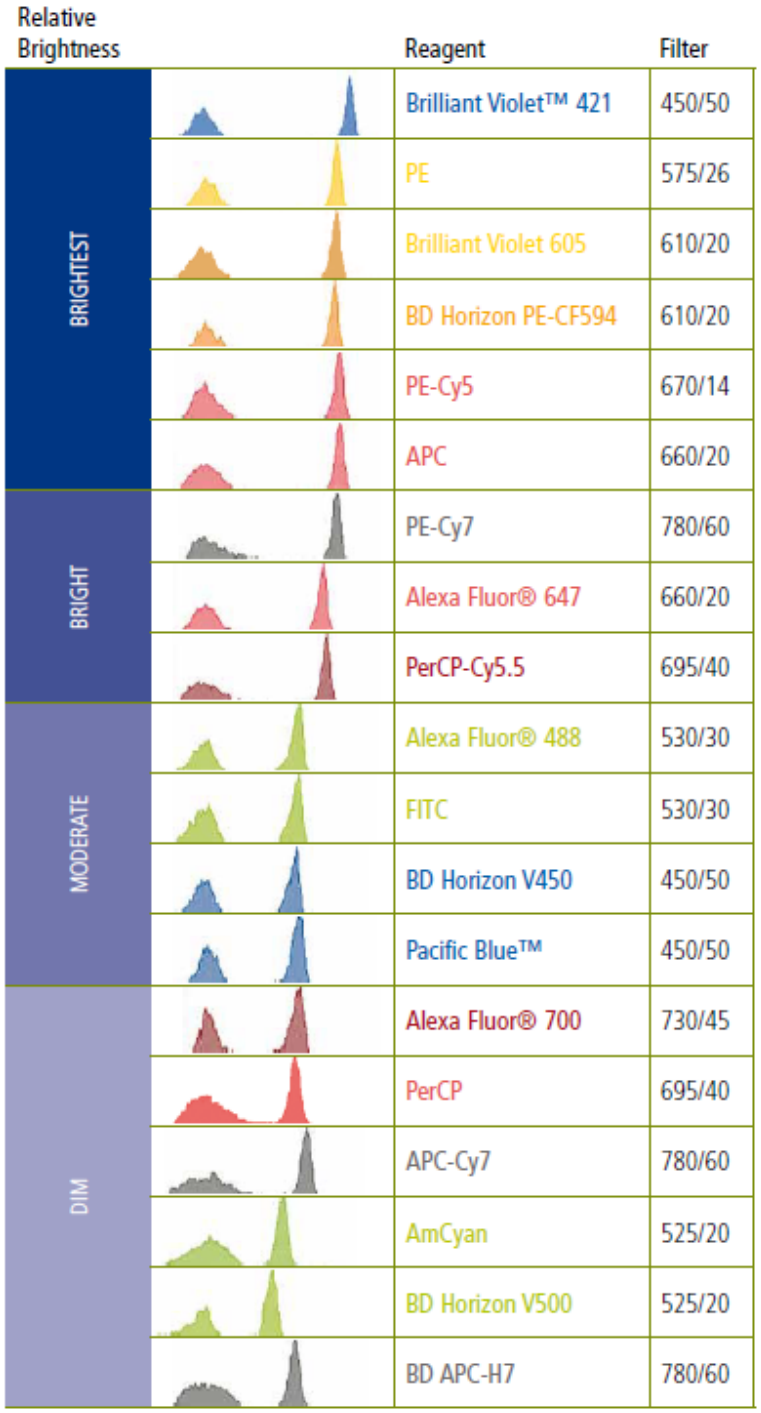
Laser configuration and power
Laser and optical alignment
Dichroic mirrors and filters
PMT sensitivity (Q)
PMT background (B)

Relative Brightness

Despite cytometer differences fluorochromes can still be grouped into

Brightest
Bright
Medium
Dim

As in this chart which is based on an average of different cytometers



Titration and PMT voltage

These can affect your stain index

Titrate your antibodies!

It is essential for all your antibodies to be correctly titrated!!

Purpose: to find the optimal concentration for each antibody to

- Maximize your stain index
- Maximize your separation of dim populations
- Minimize cost (antibodies are expensive!)

You should not depend on the manufacturer's recommendation.

They test certain cells and conditions, maybe not yours.

How to titrate

1. Serial dilution of your antibody:

    Start at 2-4x the manufacturer's recommendation

    Do 8-12 doubling dilutions

2. Add your cells of interest

    in the same conditions as your experiment

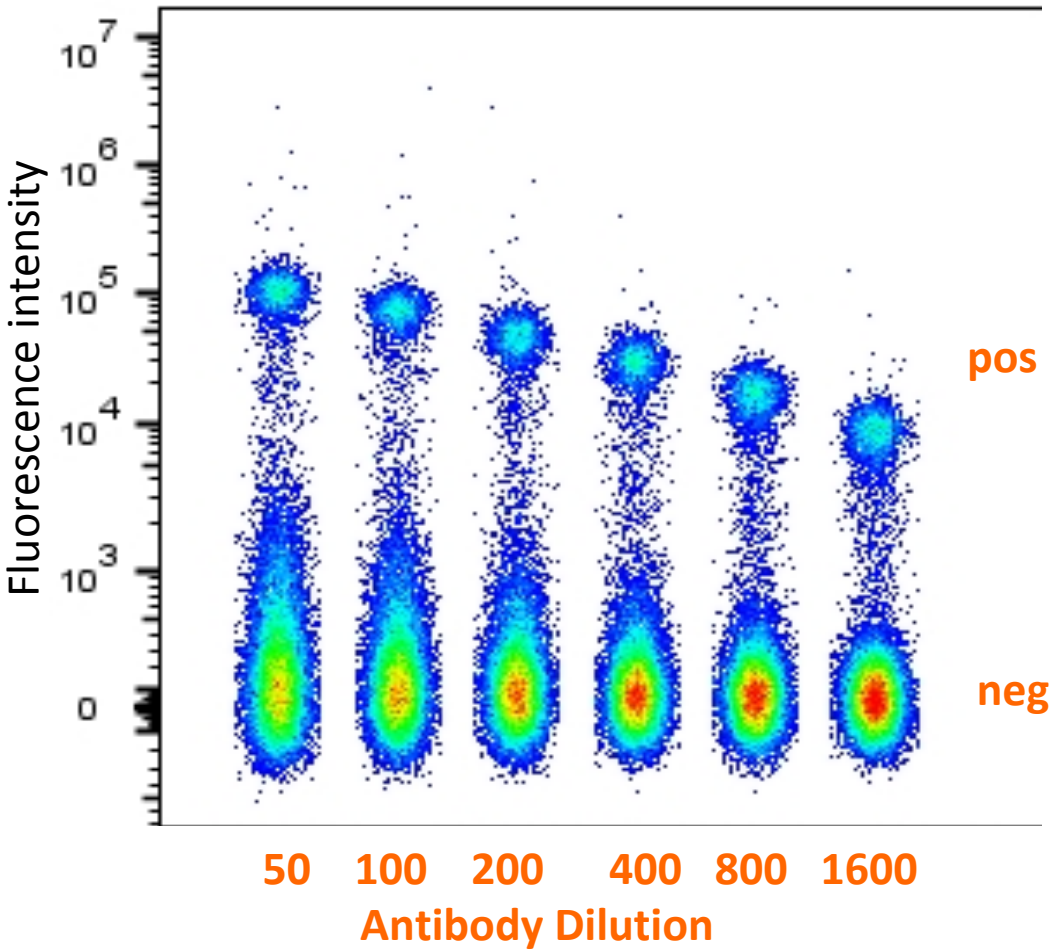
        i.e. same fixation and permeabilisation

    must have cells that are antigen positive and negative

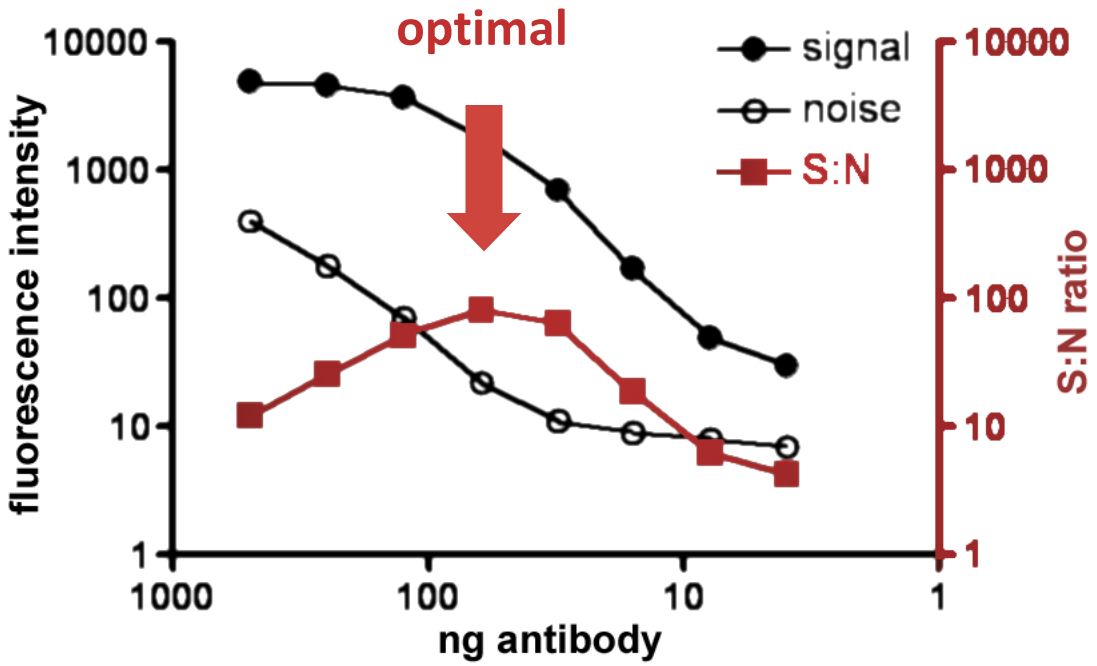
3. Wash and run on cytometer

4. Calculate stain index or signal/noise for each dilution

Titration

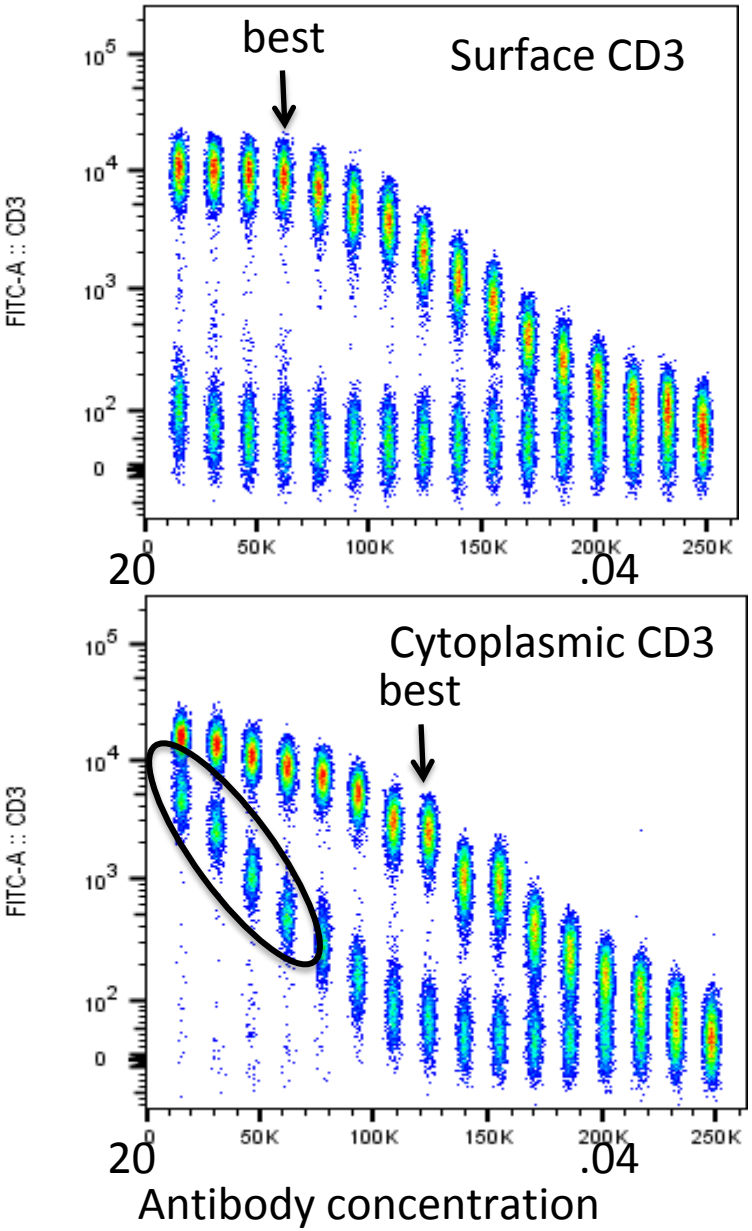

Fluorescence intensity
10^7
10^6
10^5
10^4
10^3
0
pos
neg
50
100
200
400
800
1600
Antibody Dilution

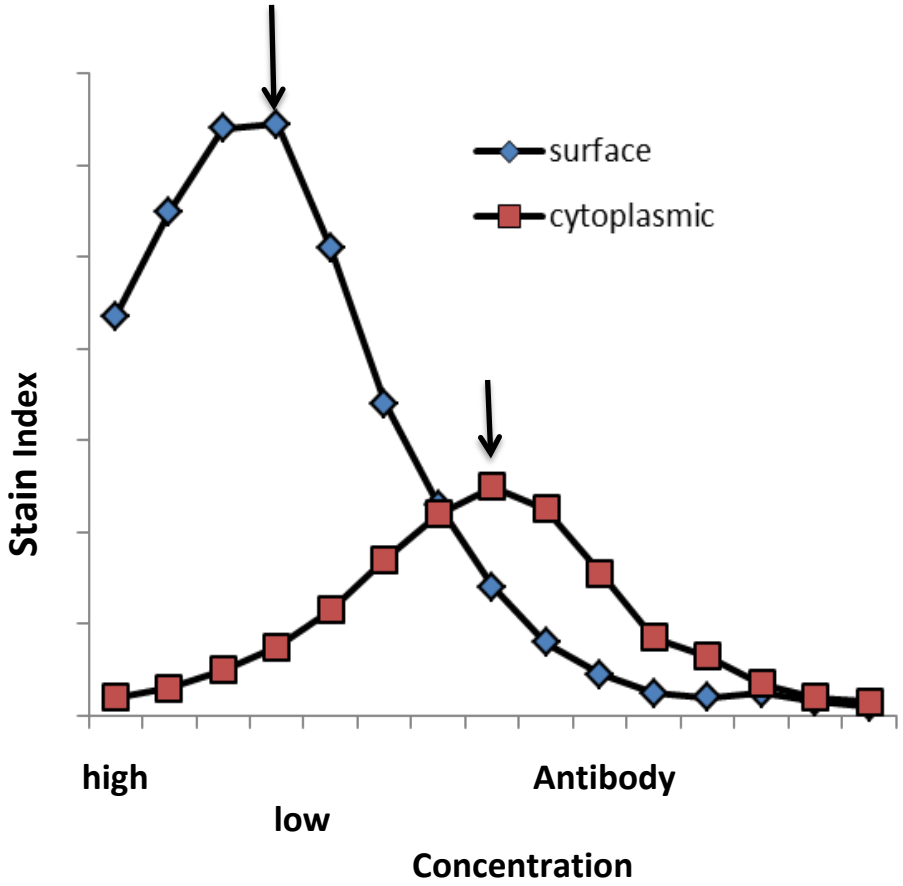
Titration



Goal is to maximize the signal (positive population) to noise (negative population) ratio.

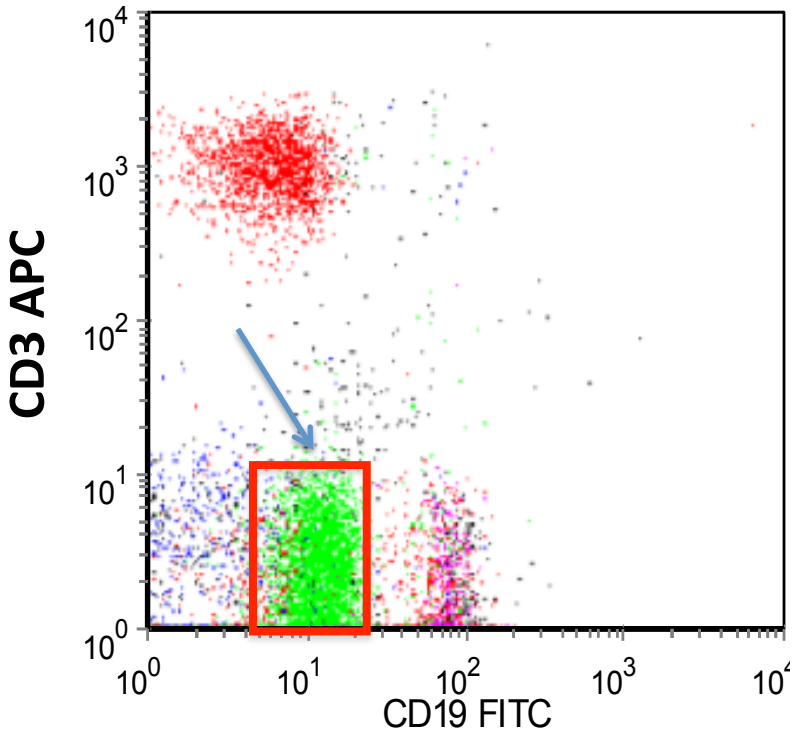
Titration CD3 surface vs CD3 cytoplasmic





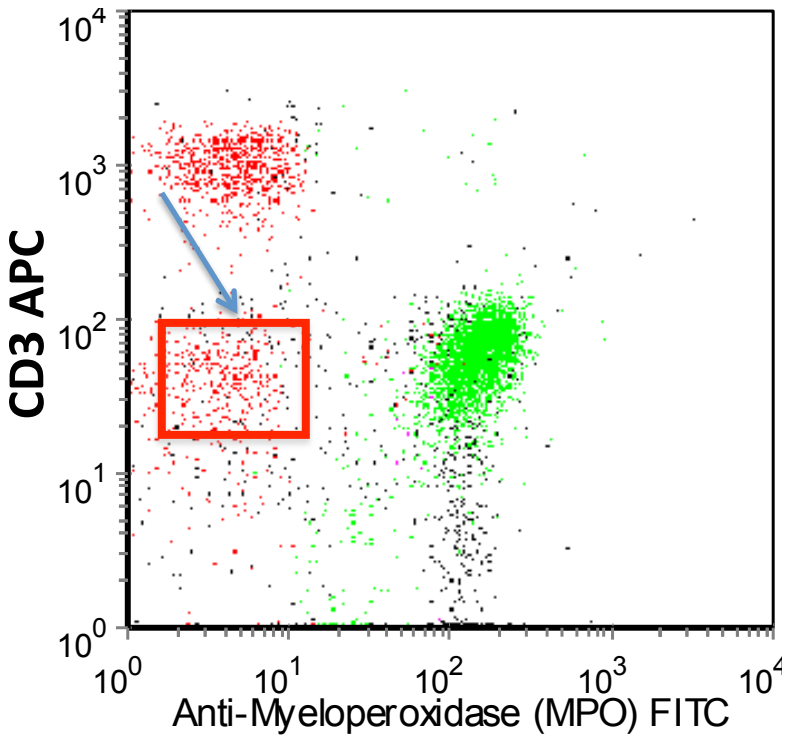
Increased Background in Fixed Cells

Surface CD3 APC (3ul):
low background



Cytoplasmic CD3 APC (3ul):
high background

due to sample processing



Titer is affected by

Staining volume (example: 100 mL)

- Number of cells (not critical up to $5x10^6$ at 100 mL volume)
- Staining time and temperature (example: 30 min RT)
- Type of sample (whole blood, PBMC, cell line)
- Scaling up to lots of cells: rough rule of thumb
    - Double the antibody concentration for every increase of every 25-50 $x10^6$ cells

PMT voltage (gain) settings

Gain or PMT voltage setting affects sensitivity

Best gain should be determined for each detector on each cytometer

Different ways to do this:

1. Voltration: Set PMT voltages to maximize Stain Index with comp beads or stained cells

2. using electronic noise and linearity criteria (CST values)

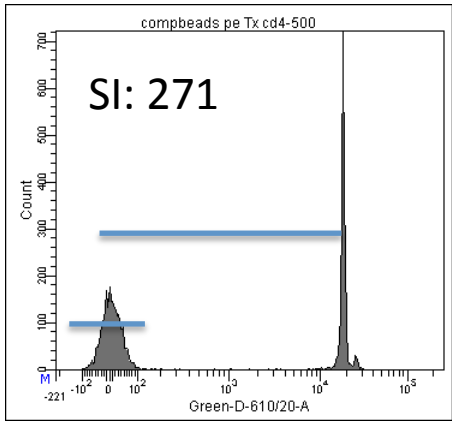
    set PMT voltage so that

        1. unstained cells have 2.5 X SD of electronic noise

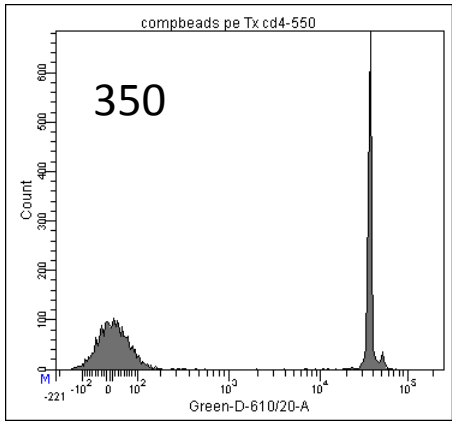
        2. positive cells not above max linearity

Voltration: choose the best stain index (SI)

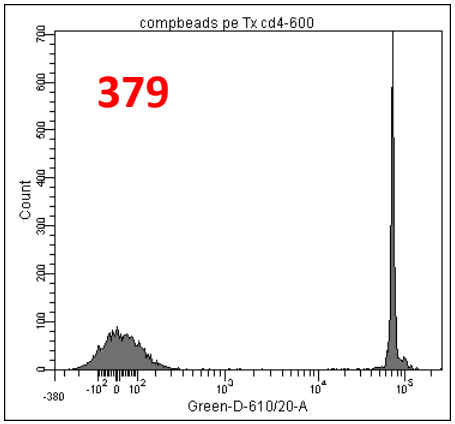
500v



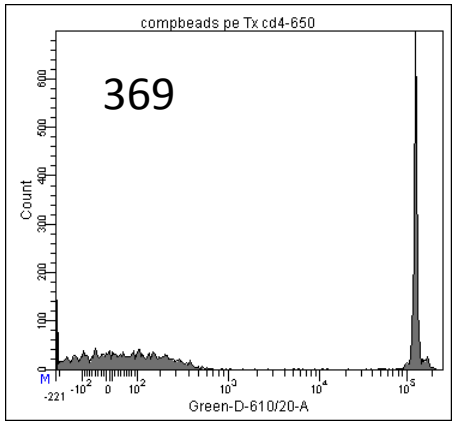
550v

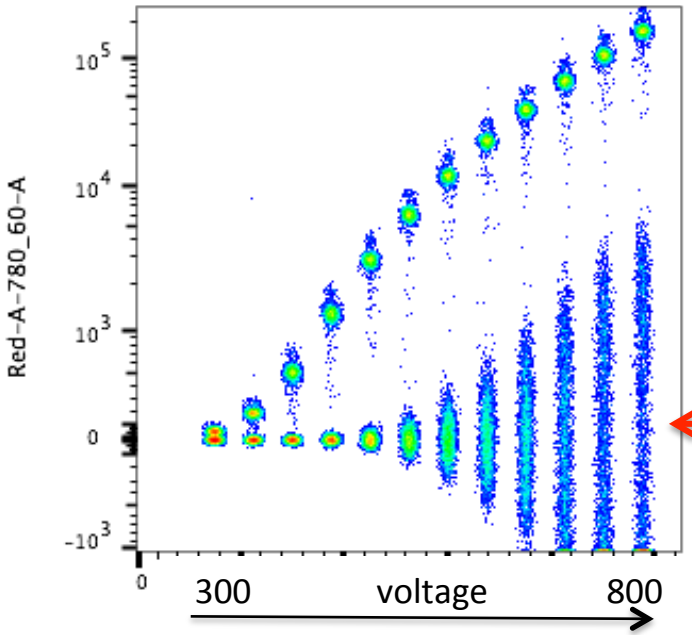


600v



650v

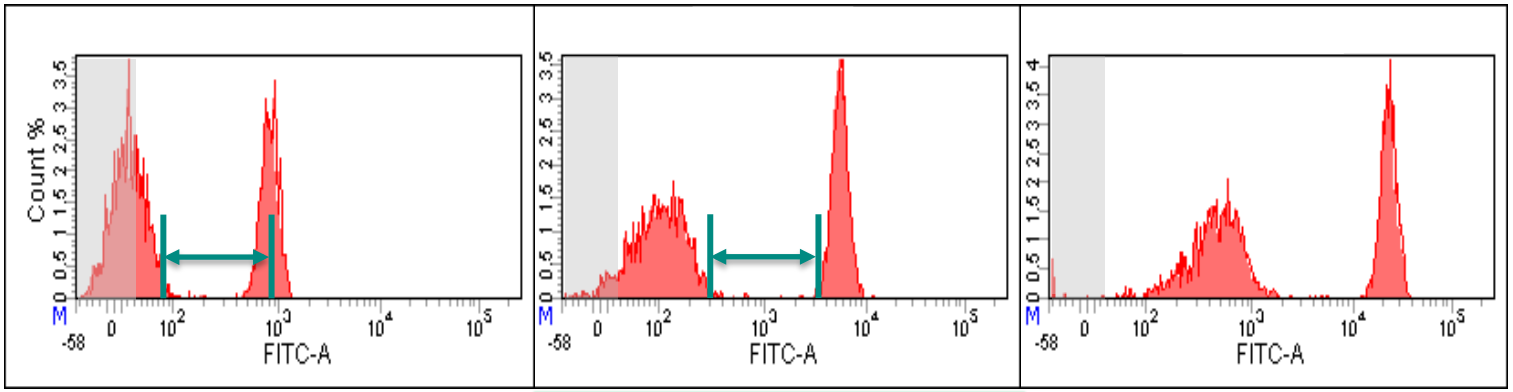




Increase of background spread at higher voltages

Voltration Example: FITC detector

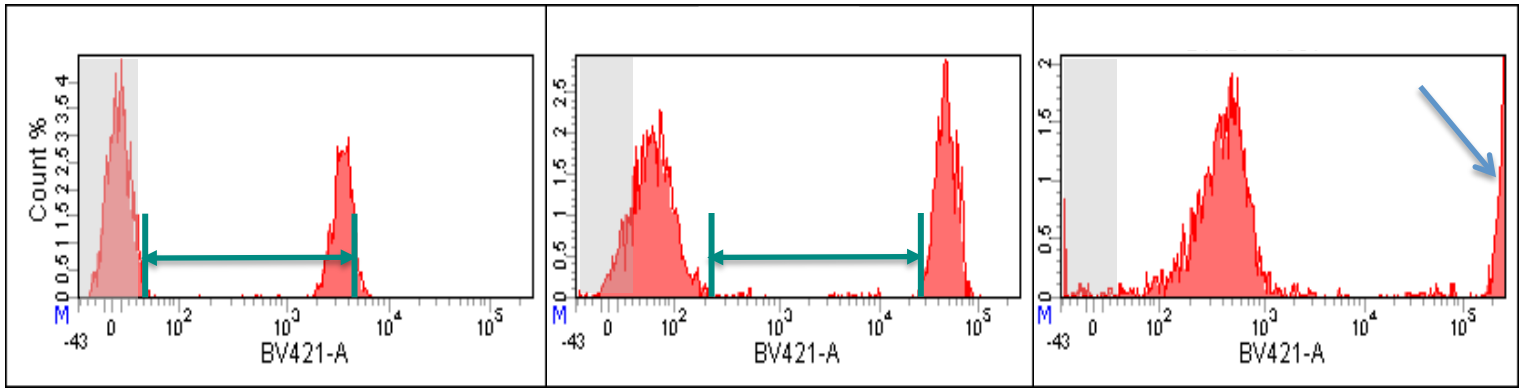
FITC Detector (SD_{EN} = 20)



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

Voltration Example: BV421 detector

BV421 Detector (SD_{EN} = 22)



PMT Voltage	350	450	550
Stain Index	125	657	>800
MFI Pos Cells	3,247	43,410	>262,143
MFI Neg Cells	4	53	368
rSD Neg Cells	13	33	207

Events should NEVER be off the top of the scale

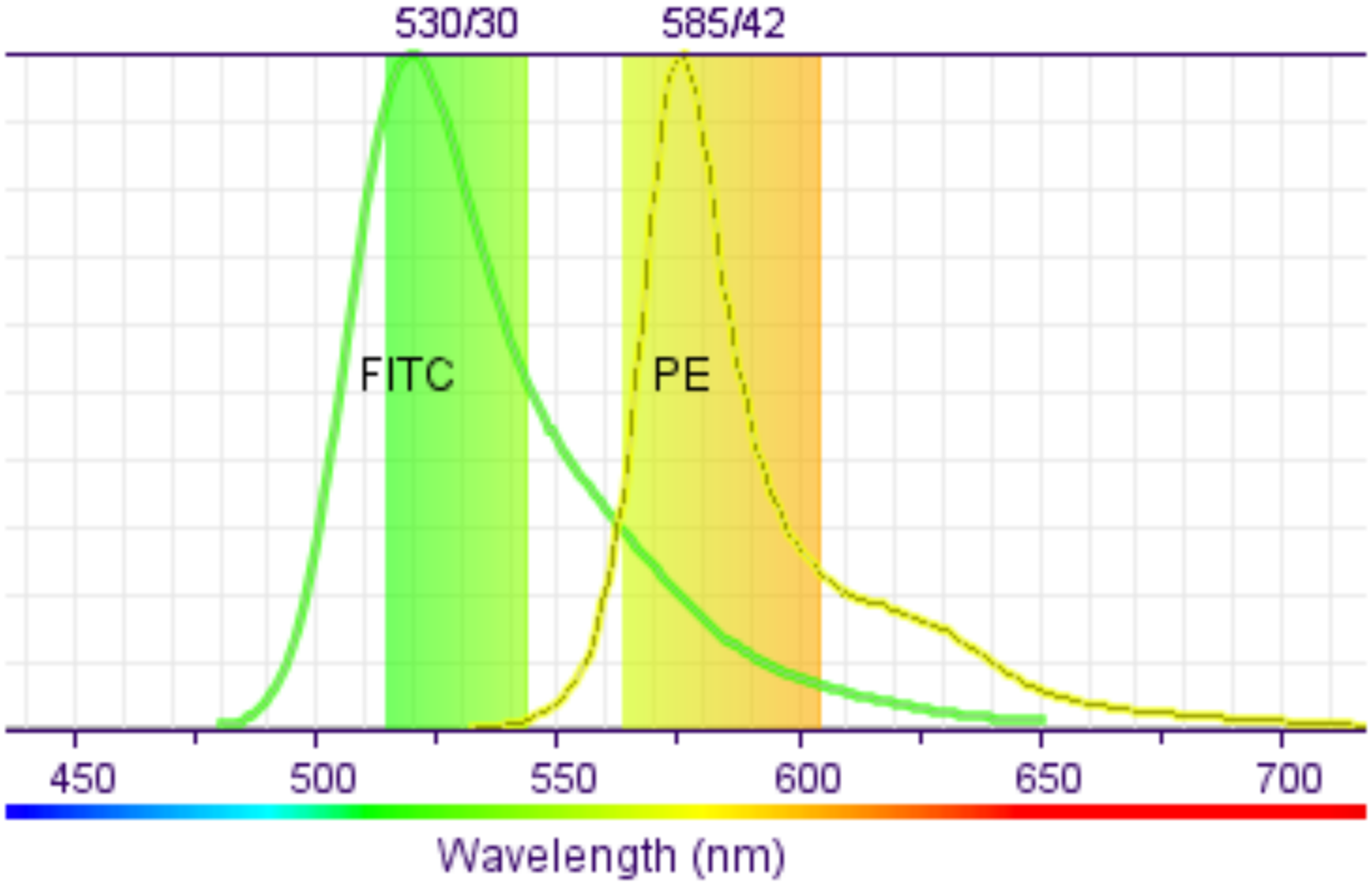
Best Gain Settings

- An issue for everyone: An entire well attended workshop was devoted to setting gains at recent CYTO meeting
- Compared several ways of best gain determination
- Most systems did not differ greatly in best gain result
- Autofluorescence affected best gain
  - Gains on lymphs or beads not the same as highly autofluorescent cells
  - may need to determine best gain separately on very autofluorescent cells

Spectral Overlap, Spillover and Compensation

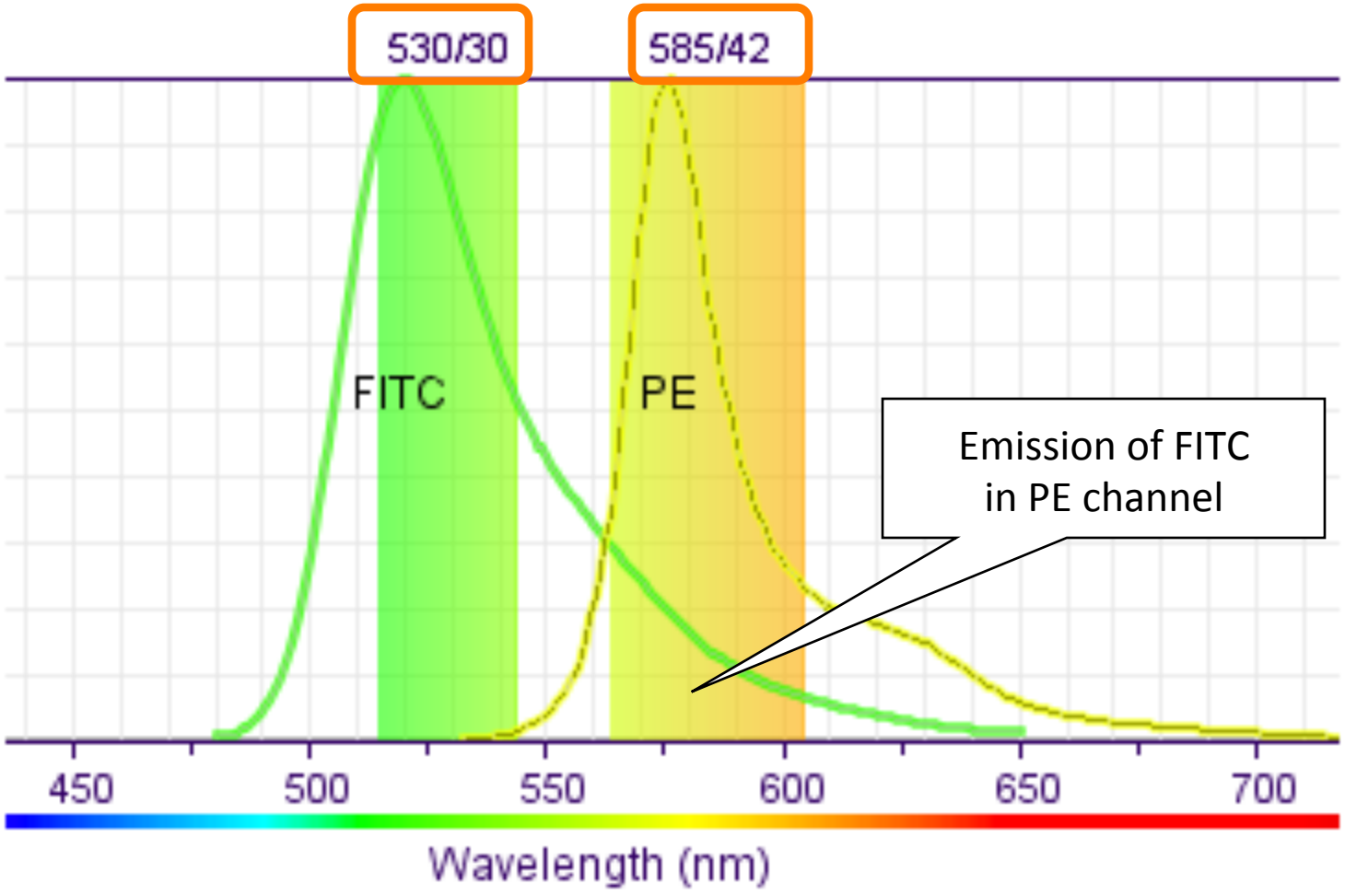
Fluorochrome Emission Range

Fluorochromes absorb and emit over a range of wavelengths specific to each fluorochrome



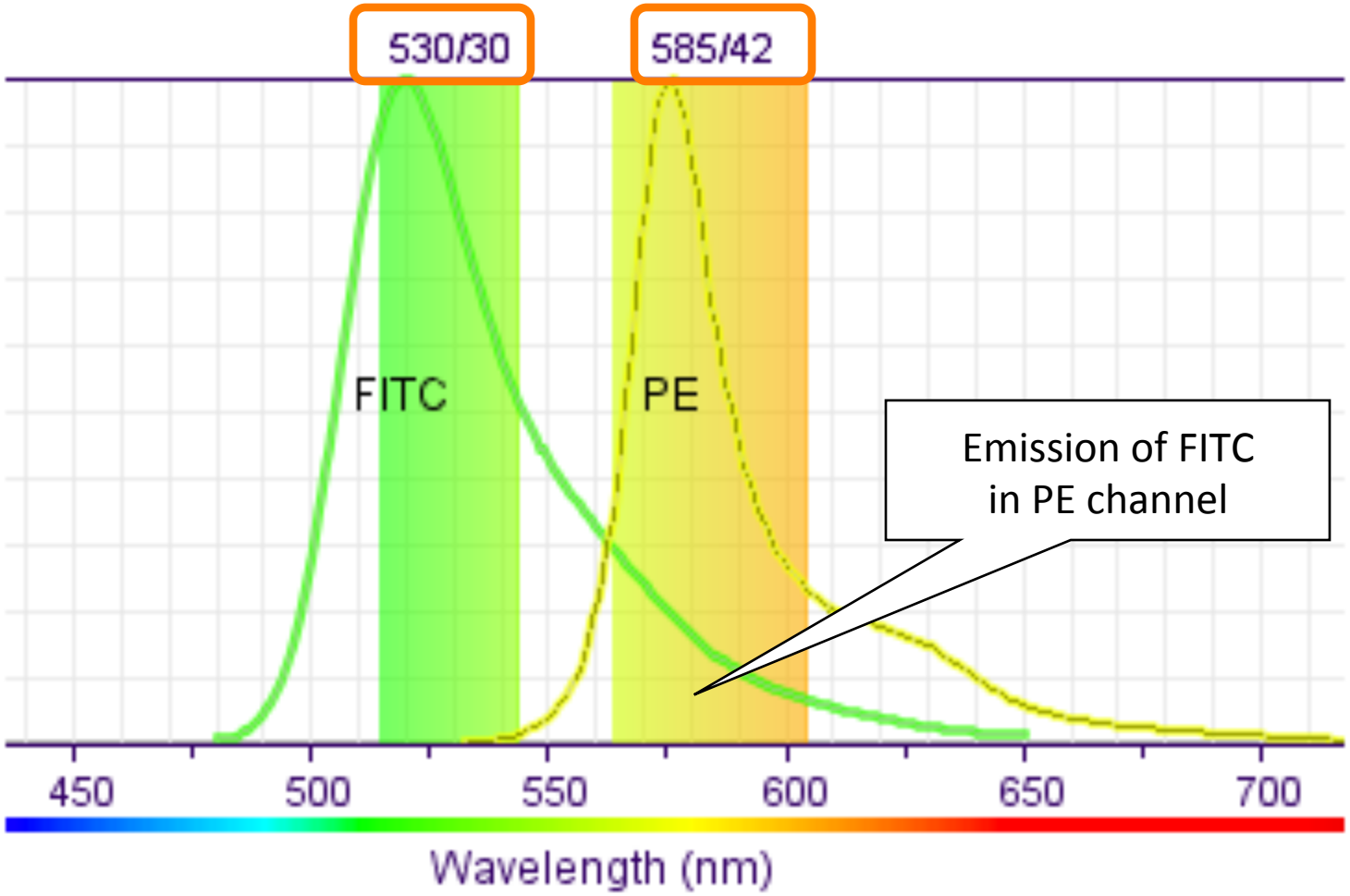
Fluorescence Spillover

These are the optical filters in which each fluorochrome is detected:
the longer wavelength emission from FITC is seen in the PE detector



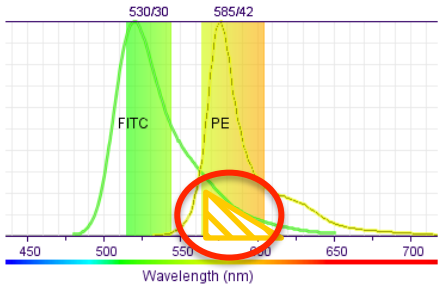
Fluorescence Spillover

Thus part of the emission measured in the PE detector is due to FITC emission
But we need to measure each fluorochrome separately



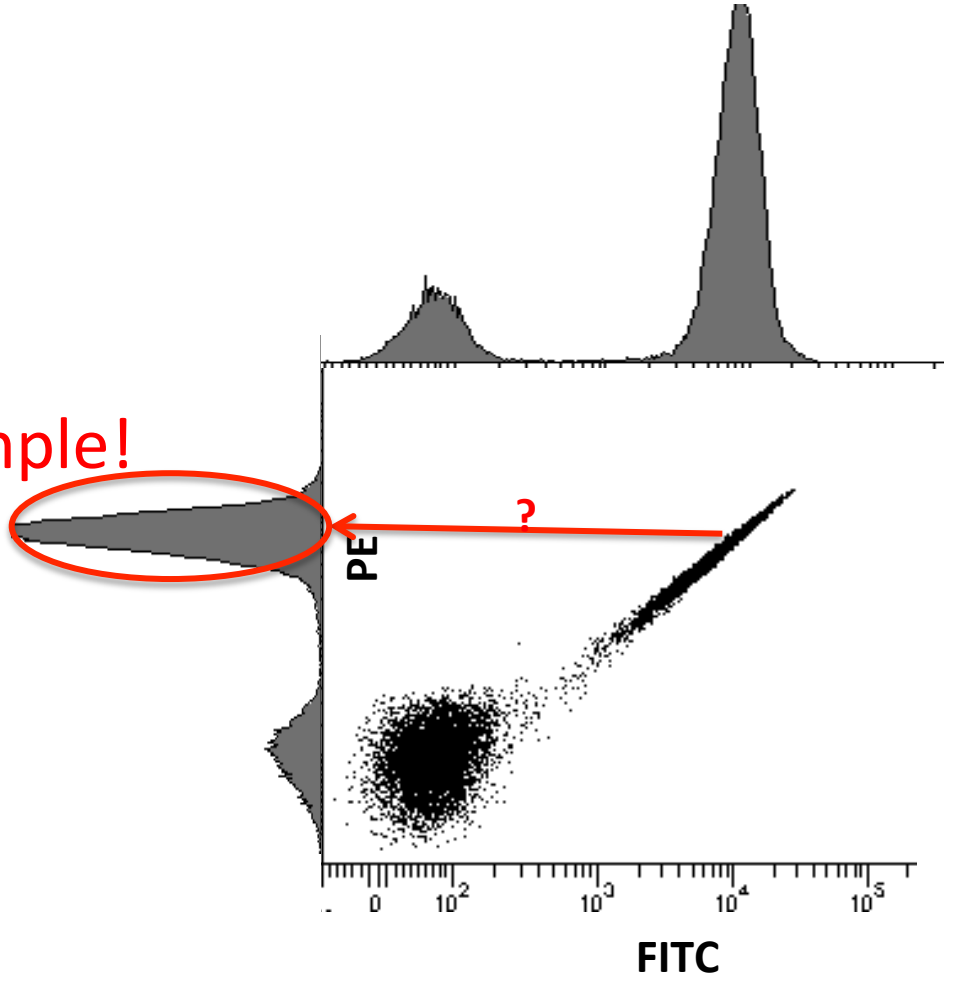
The Spillover Effect: FITC into PE

This is a single color control stained **only** with FITC.



There is no PE in this sample!

The PE signal we see is FITC fluorescence coming through into the PE detector.



So what do we do?

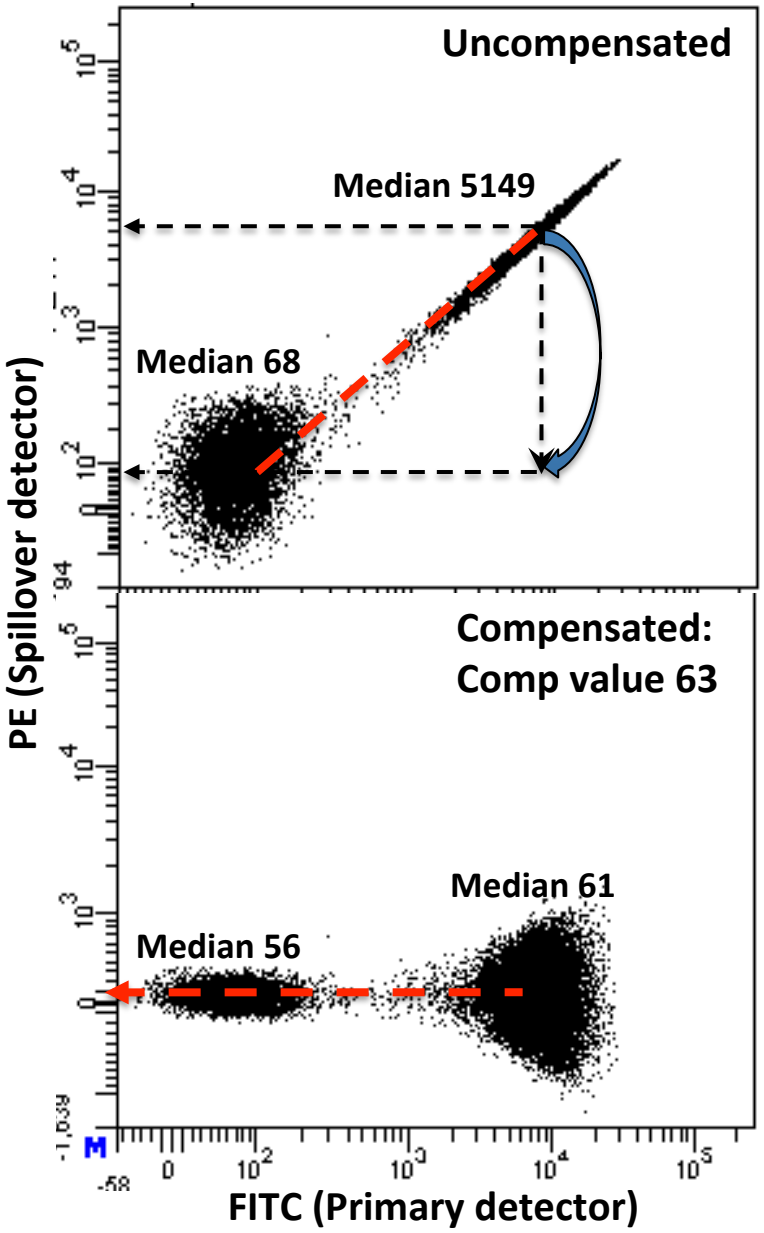
We compensate!

Compensation is a mathematical correction that is applied to correct for spillover from other fluorochromes so that:

The true PE signal =

observed PE signal – contribution (x%) of FITC signal

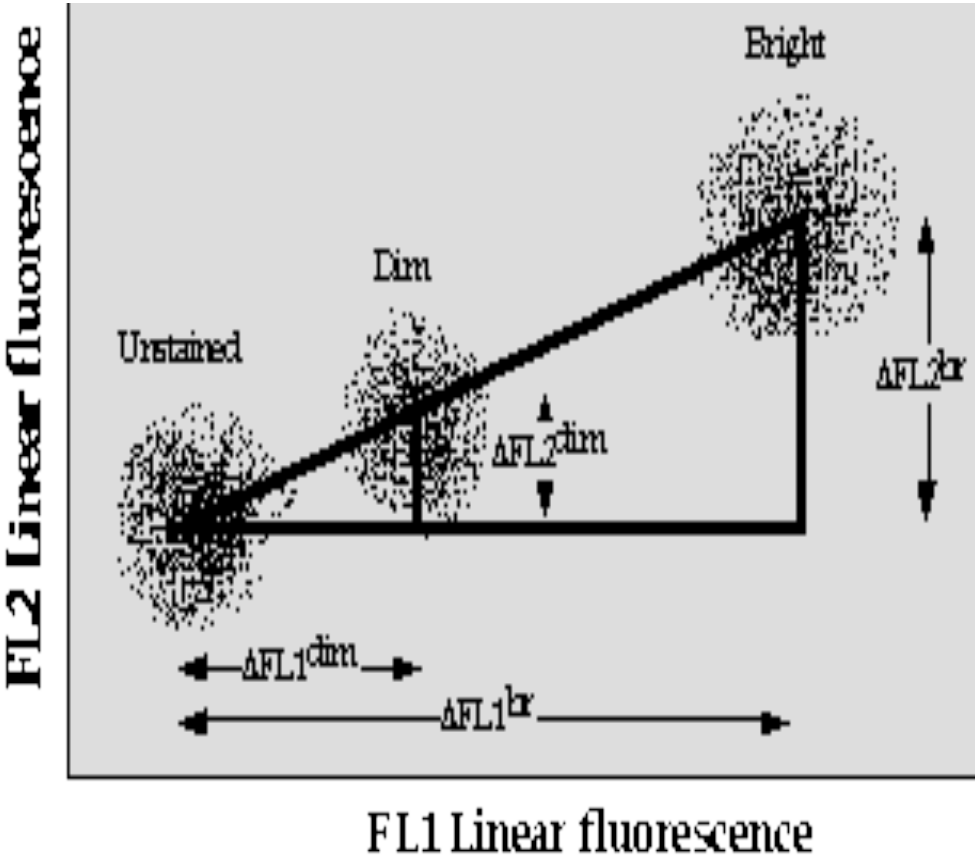
Spillover is corrected by Compensation



The median intensities
of the positive and negative populations
in the each channel are measured.

Compensation is applied so that
the PE median of the FITC positive
population
is the same as
the PE median of the FITC negative
population.

How is Compensation Calculated?



based on the slope of the line between the positive and negative medians

Actual calculation complicated:

- uses inverted spillover matrix
- matrix algebra

Compensation is not a simple percentage:

- values of over 100%
- are possible
- not necessarily a cause for concern

Compensation

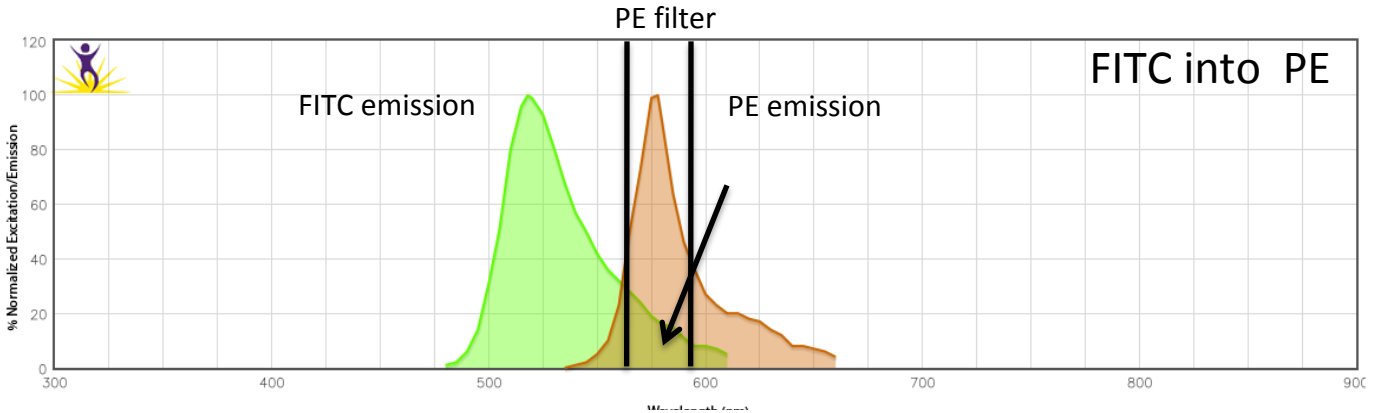
- The contribution of light signal from all other fluorochromes must be measured using single color controls for each color in the mix.

- **Compensation is more accurate using automated software than manual,** because the automated corrects for spillover of all fluorochromes into all channels simultaneously**.**

Three Sources of Spillover

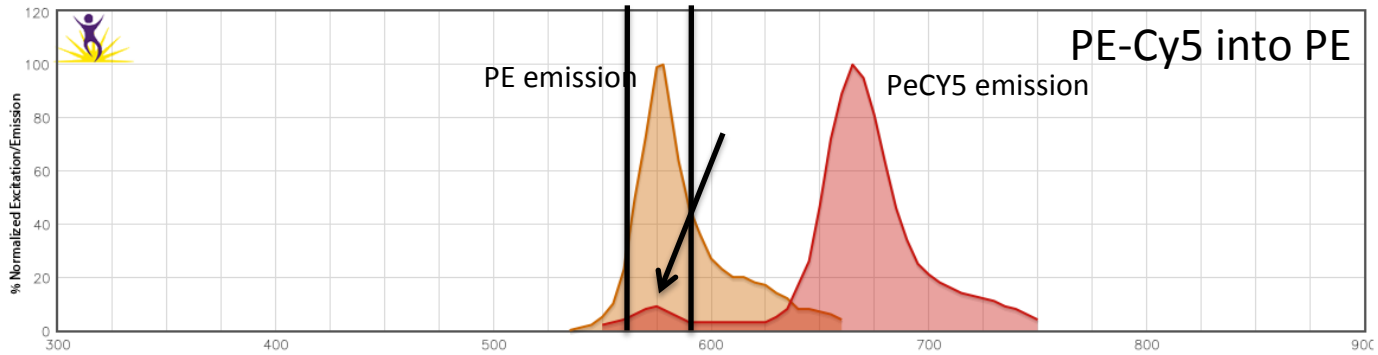
1

Adjacent spectra



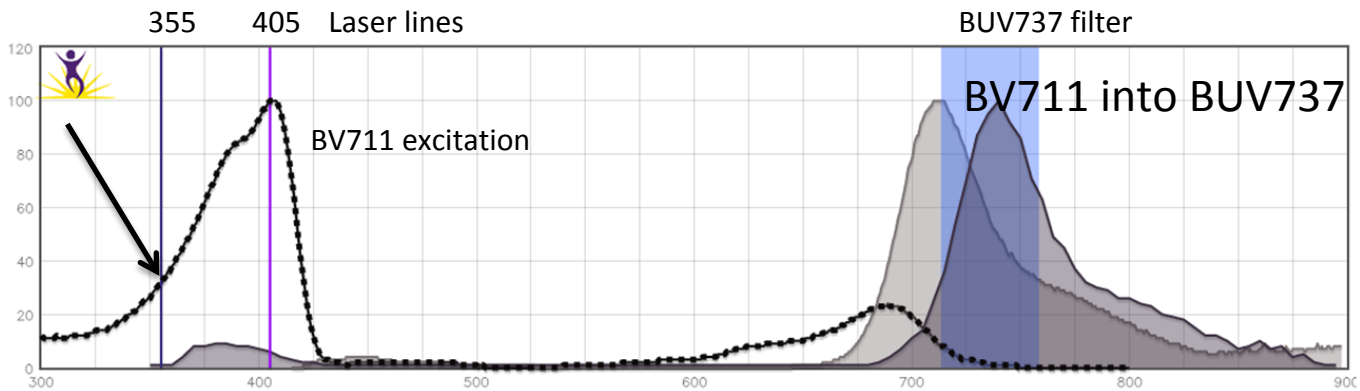
2

Tandems and their bases

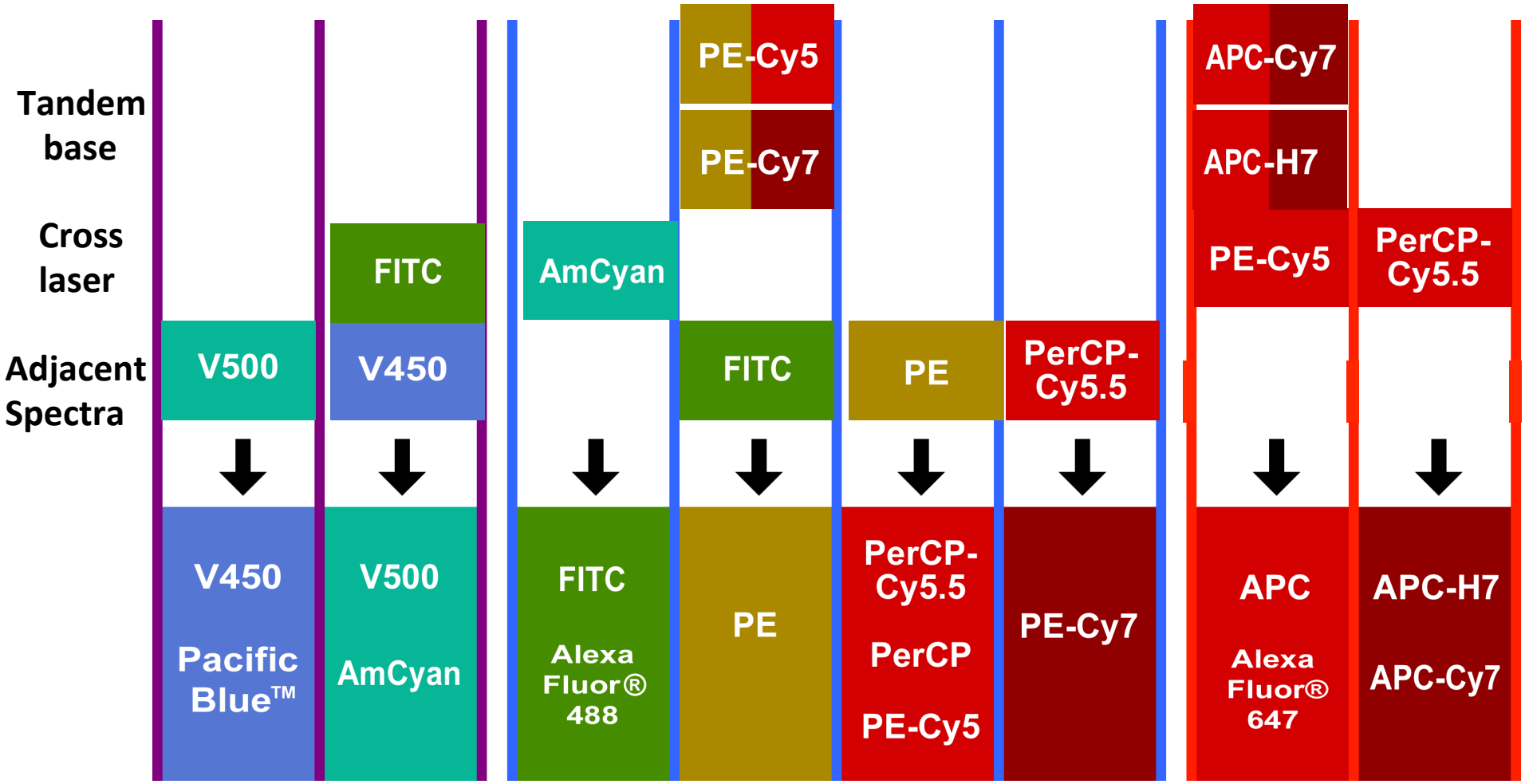


3

Cross laser excitation

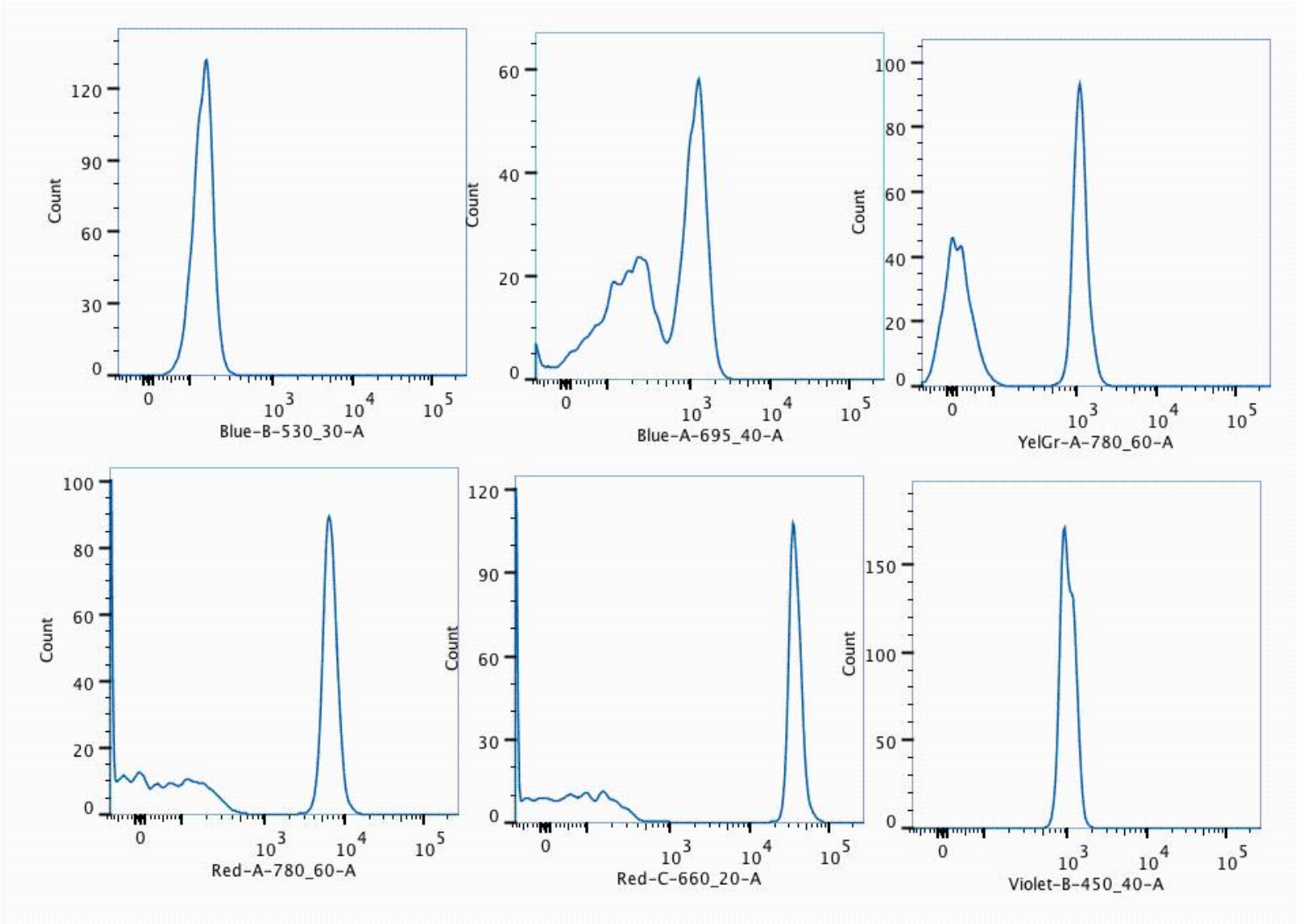


Who spills into what?



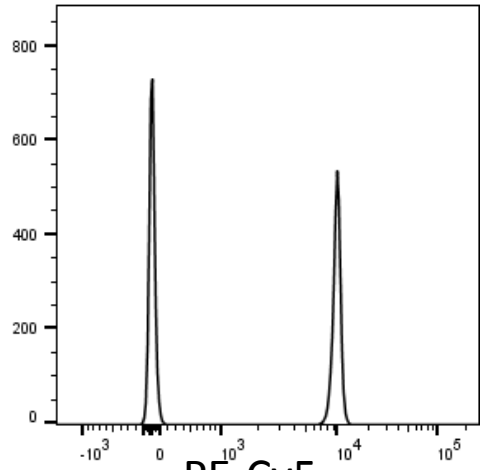
APC single color

Look where it spills!! There is only APC in this tube

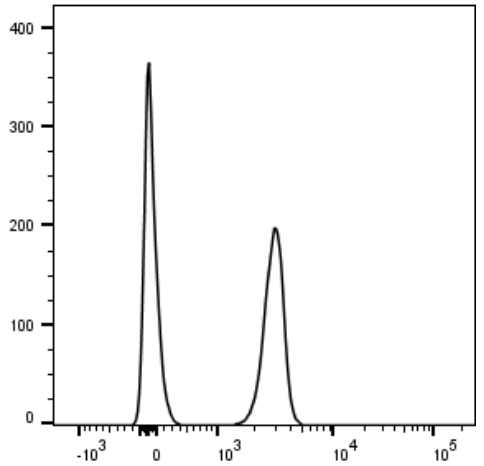


PE-Cy5 Single Color

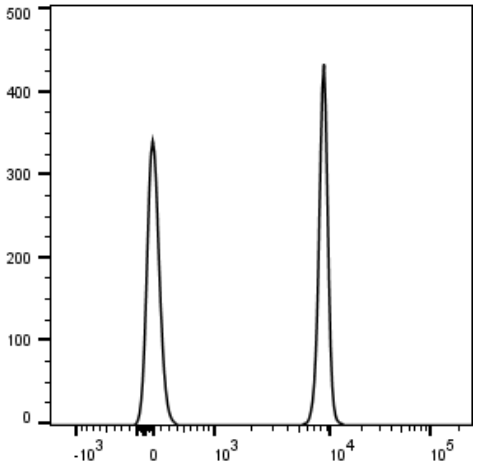
Look where it spills!! There is only Pe-Cy5 in this tube
Which categories of spillover apply here?



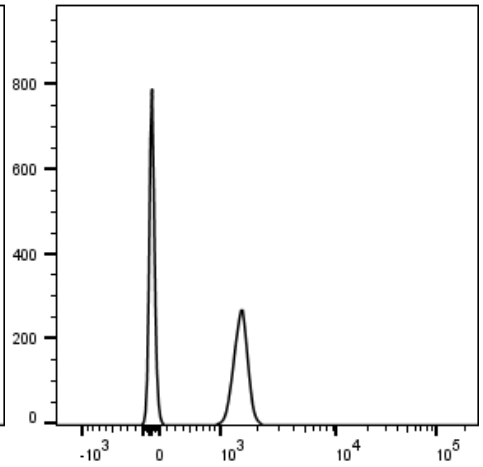
PE-Cy5



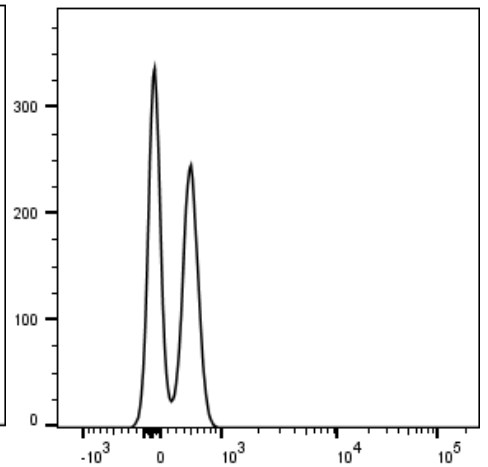
PE-Cy7



APC



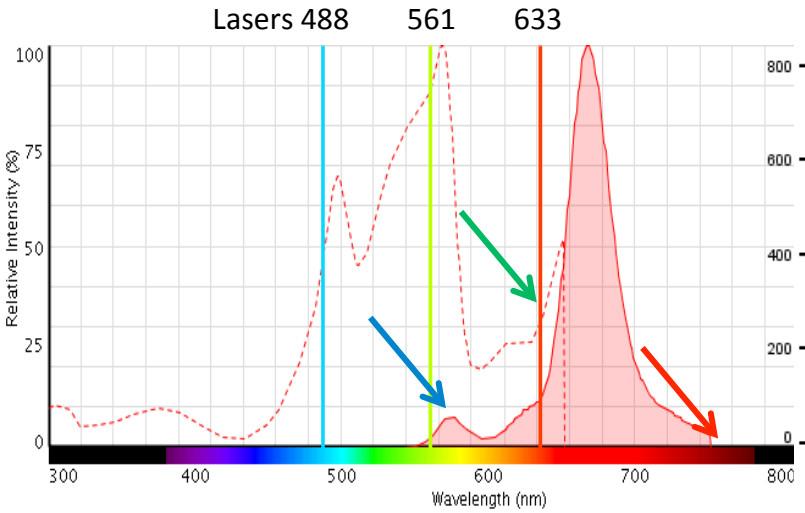
Alexa700

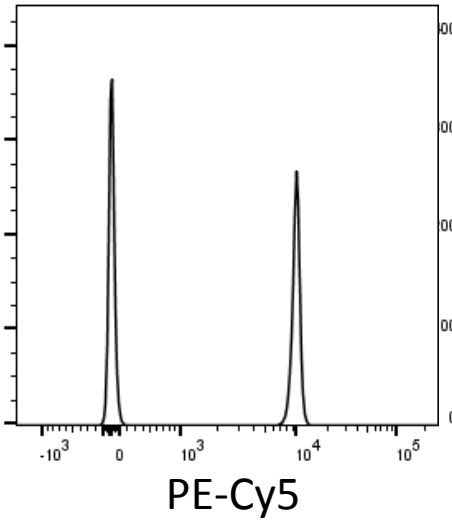


PE

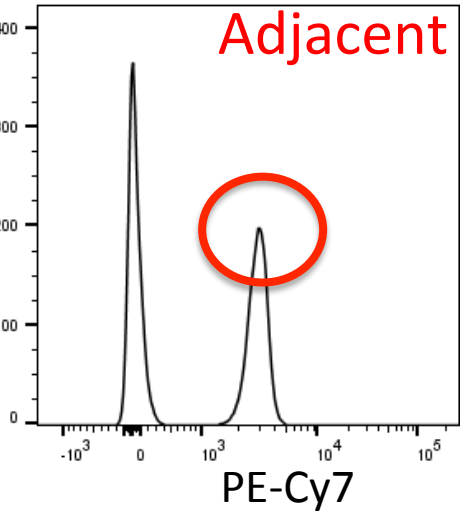
Spillover: PE-Cy5 Single Color

You can a good idea of where there will be a spillover problem by looking at the excitation and emission spectra

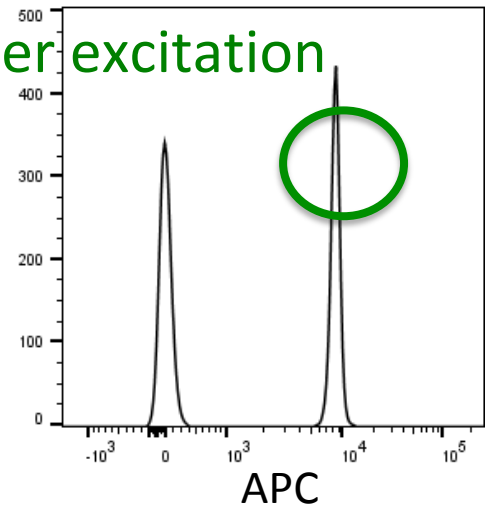


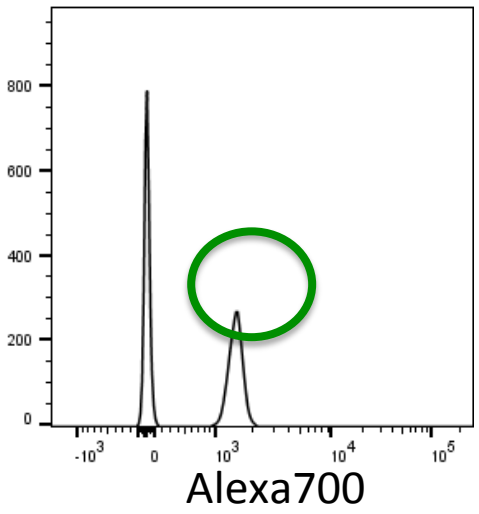


Adjacent spectra

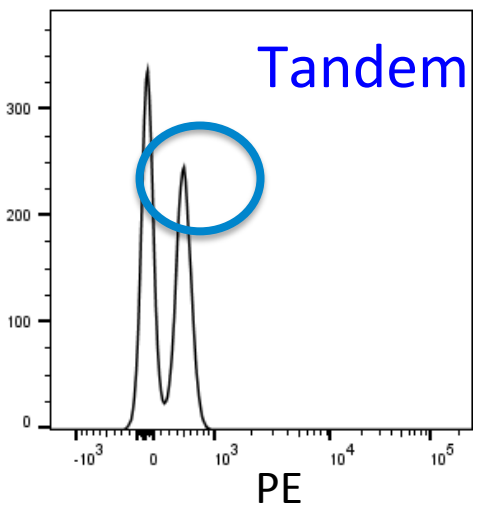


Cross laser excitation





Tandem base



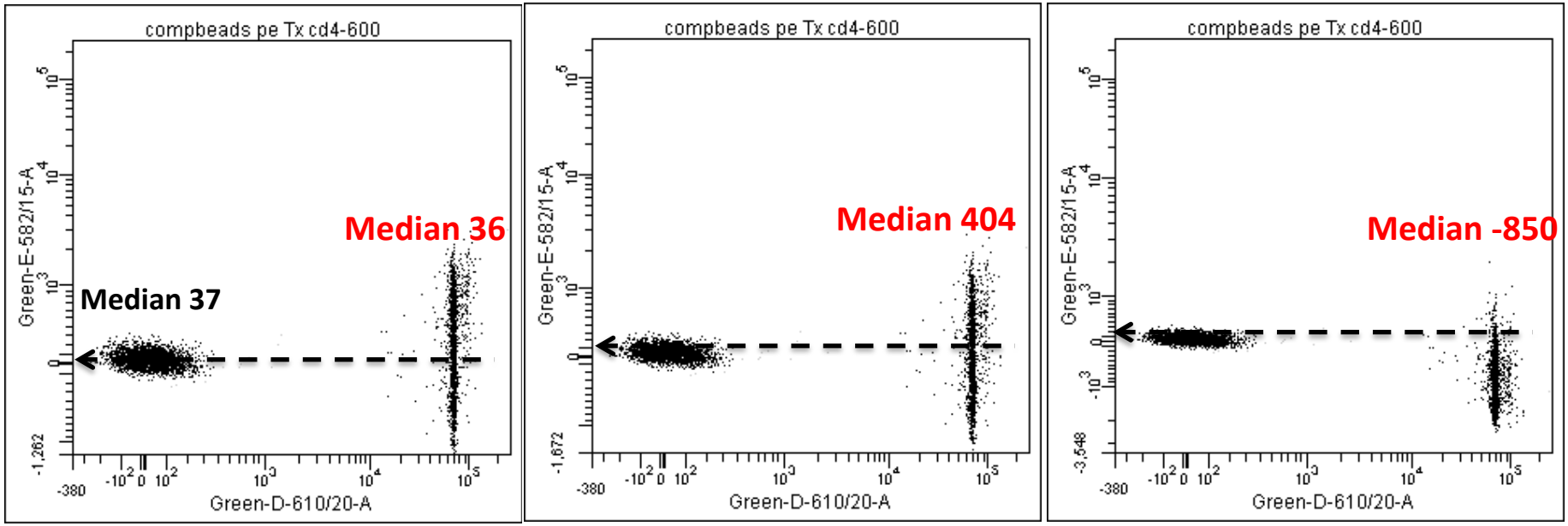
Over and under compensation

PE-Texas Red single color control

Correctly compensated
24.02%

Undercompensated
23.7%

Overcompensated
25.3%

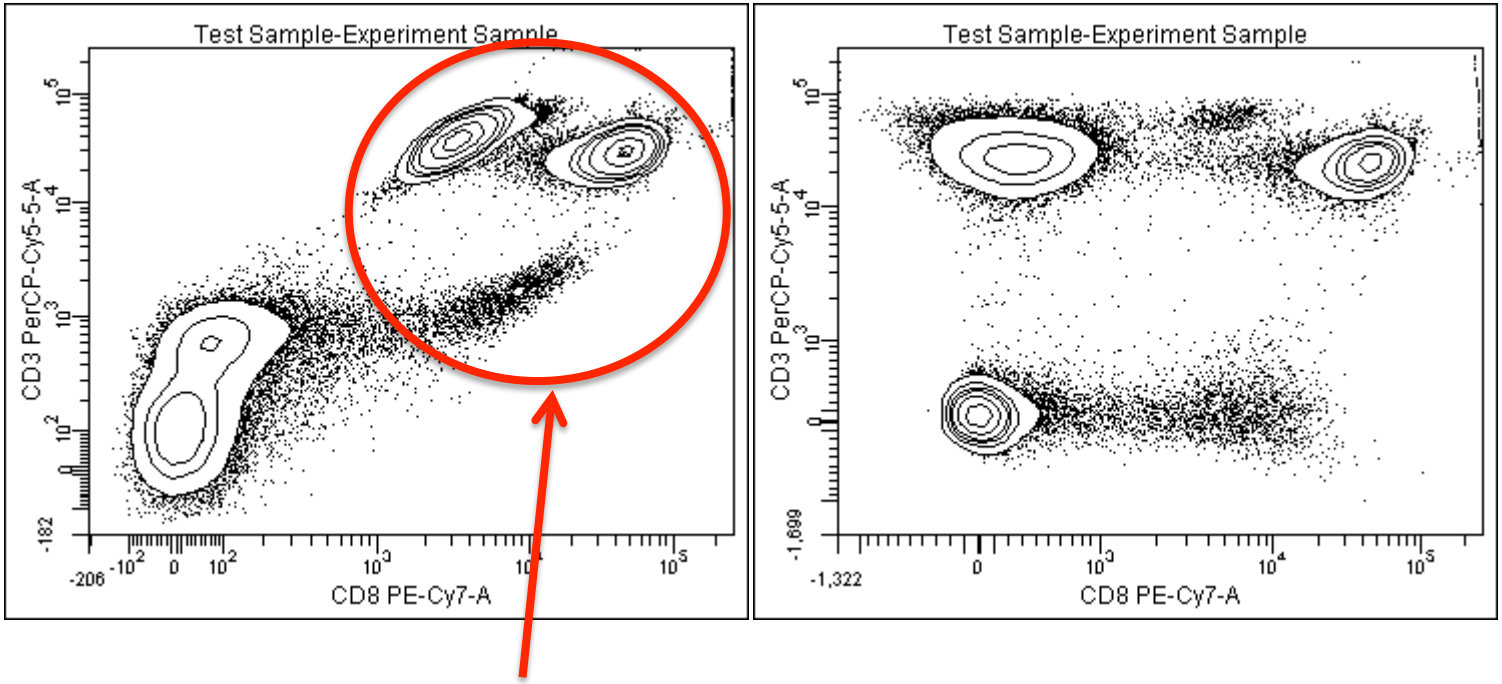


Never adjust a compensation 'by eye': Always use median statistics

Correct Compensation: the end result

uncompensated

compensated

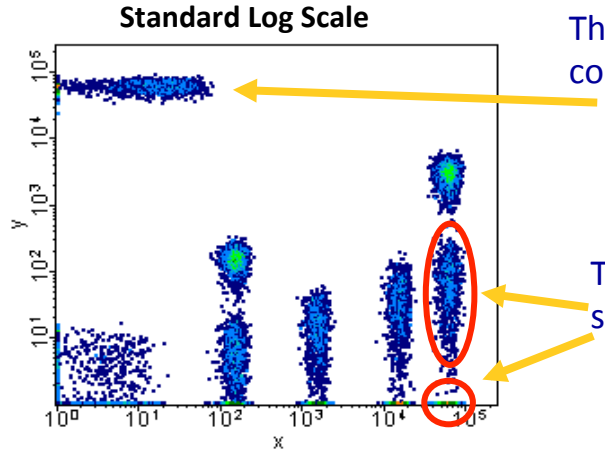


Looks like 3 double positive populations!

But it's not the case when compensated

The Bi-exponential Scale:

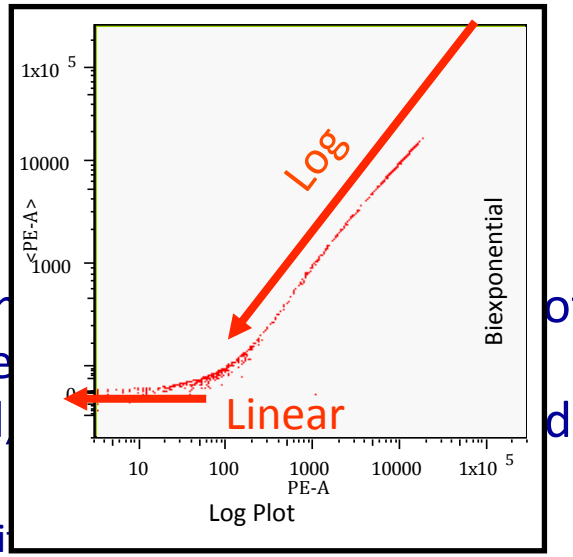
The best way to look at compensated (or uncompensated) data

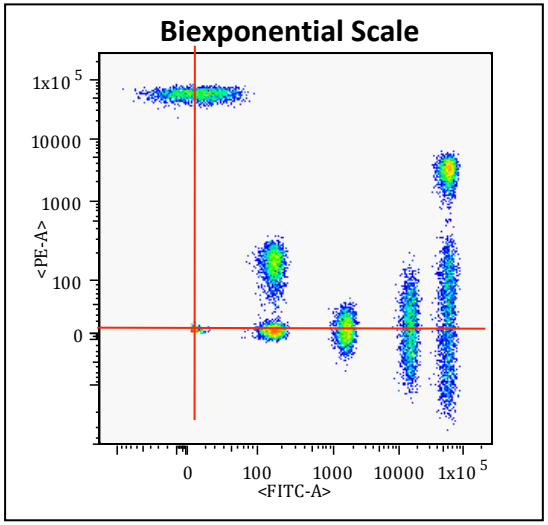


This population "looks"under compensated.

These look like two separate populations.

Visualization of compensated data is greatly improved using the biexponential scale.



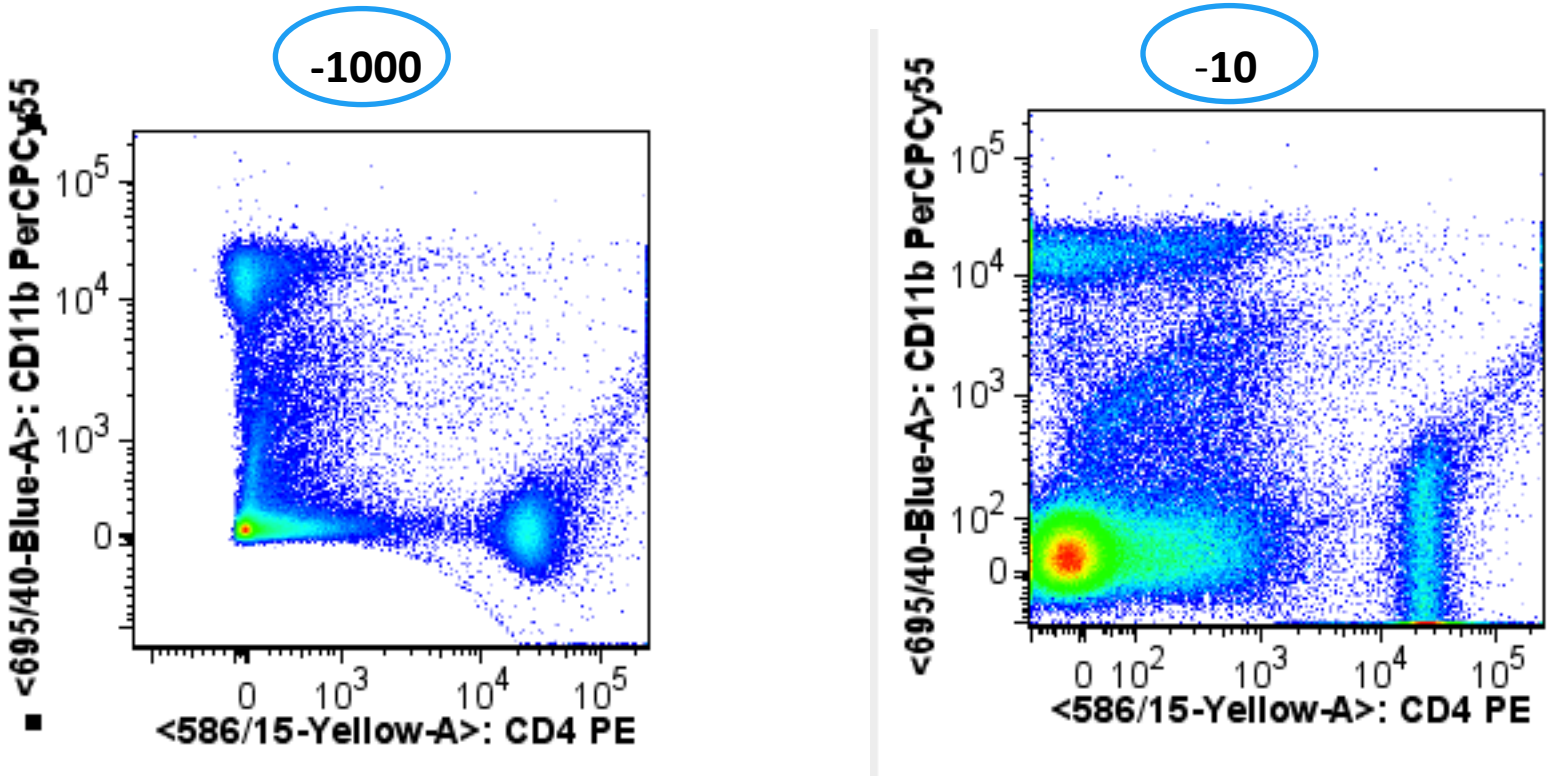


This example of the sar of the Biexponential Scale scale on the upper end, d symmetrical about the

- Compensated single posi
- All populations are visible.

SCALING OPTIONS

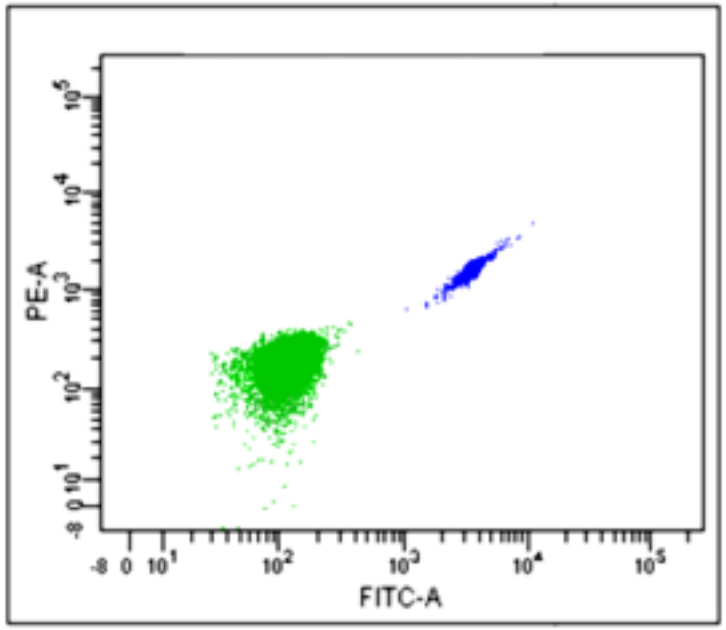
You can choose the scaling for bi-exponential to show more or less of what is below 0

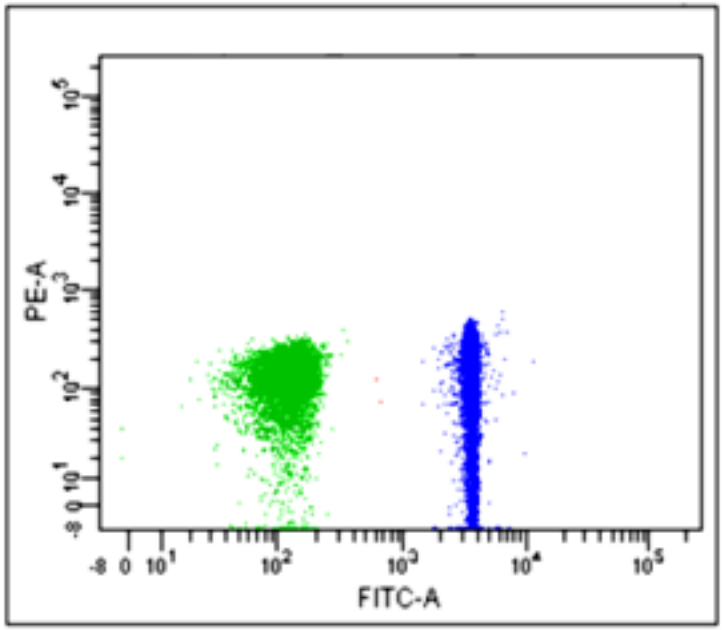


Always show axis ticks on plot, **include '0'** if you are using bi-exponential data display

How to do Compensation

You must have a good single color control for each color used!





Single color controls must have

a negative population and a population positive in one channel only

3 Rules for Compensation

1. Compensation control antibody must be the same as the one in the experiment mix

2. The positive control must be at least as bright as the experimental sample

3. The negative and positive populations for each single color control must have the same autofluorescence.

A nice discussion: http://flowjo.typepad.com/the_daily_dongle/tips_and_tricks/

Rule #1: single color antibody must be the same as mix

Single color control antibody must have the same **spectral characteristics** as the antibody in the mix.

Similar fluorochromes have different spillover values

FITC is not Alexa 488 is not GFP!

Same antibody rule is critical for Tandem reagents:

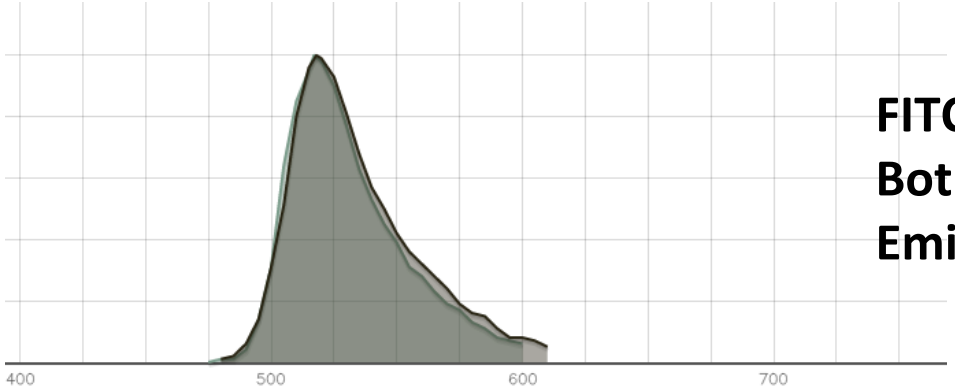
Different lots of the same antibody have different spillover

Tandems can degrade over time or in light

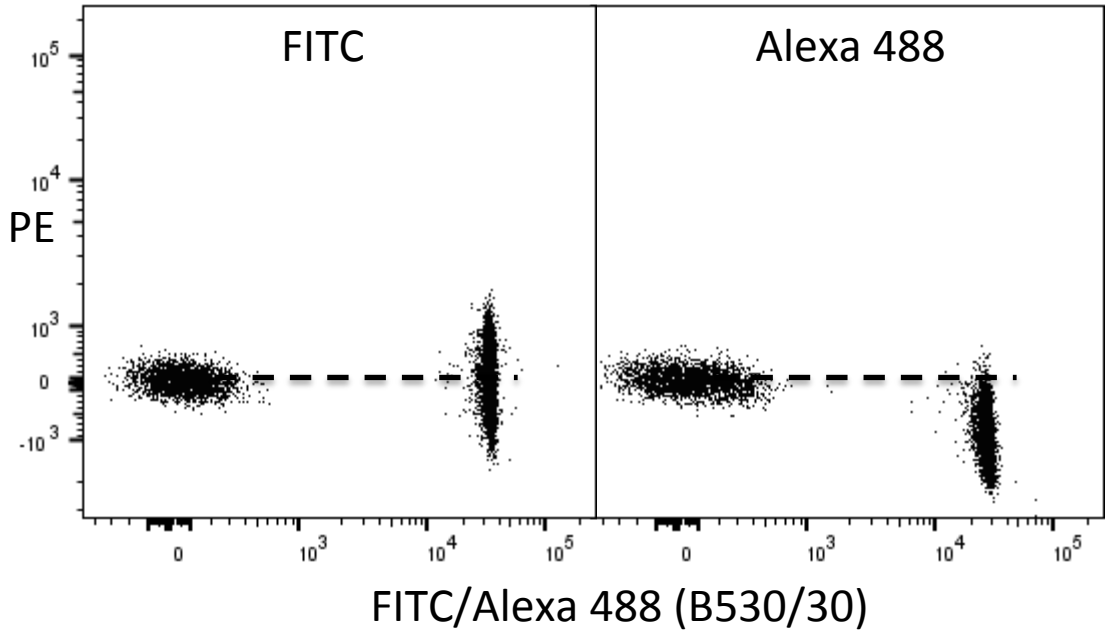
Degraded tandems will have more spillover from the base

Rule #1: FITC vs Alexa 488

When Green is not just Green



FITC and Alexa 488
Both measured in the green channel
Emission spectra look almost the same



Not in the eyes of the cytometer!

Compensation calculated using a FITC single color results in overcompensation when applied to Alexa 488

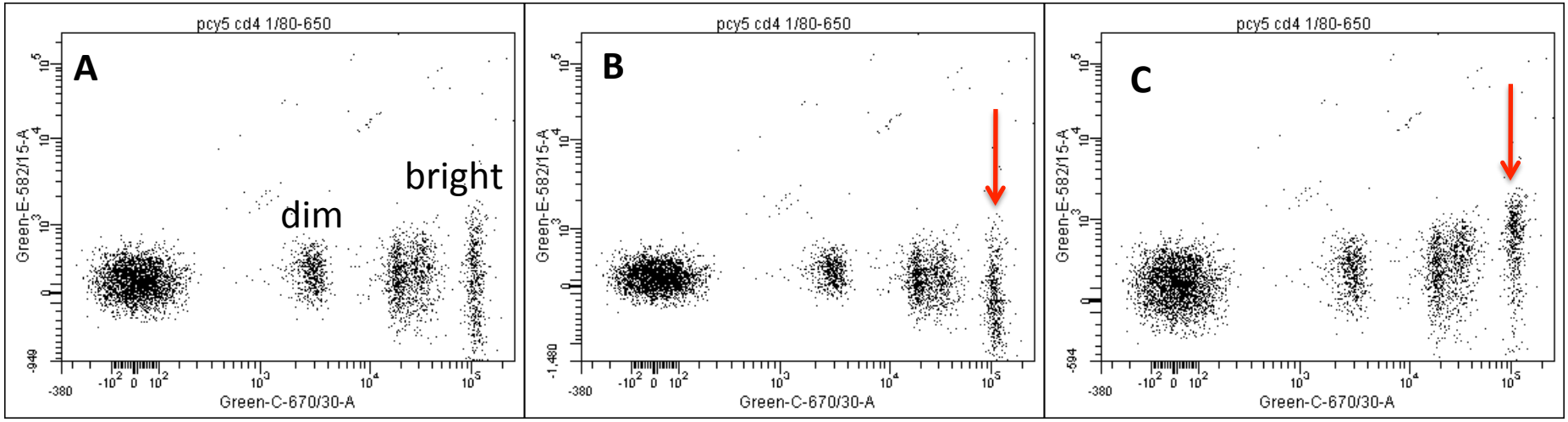
Rule #2: the single color must be as bright as the mix

Small errors in compensation using a dim single color control can result in large compensation errors with bright populations

Correctly compensated

Overcompensated

Undercompensated



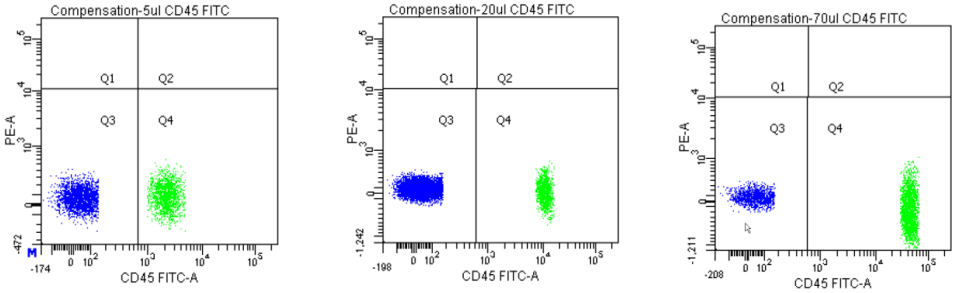
The most positive populations, whether in the single color or mix,

must be within the range of linearity of the detector

Bright signals provide more accurate compensation values

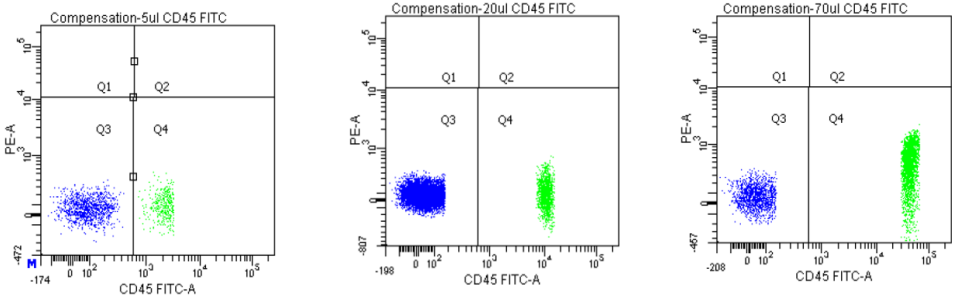
Correct compensation

Bright population used for compensation



Incorrect compensation

Dim population used for compensation



	Difference in MFI (Y-Axis) negative vs positive		
Correct compensation	8	5	7
Under-compensation	4	85	245

Rule #3: Positive and negative must have same autofluorescence

Can use antibody stained cells or beads as compensation controls

BUT the negative control must have the same autofluorescence

- beads with beads

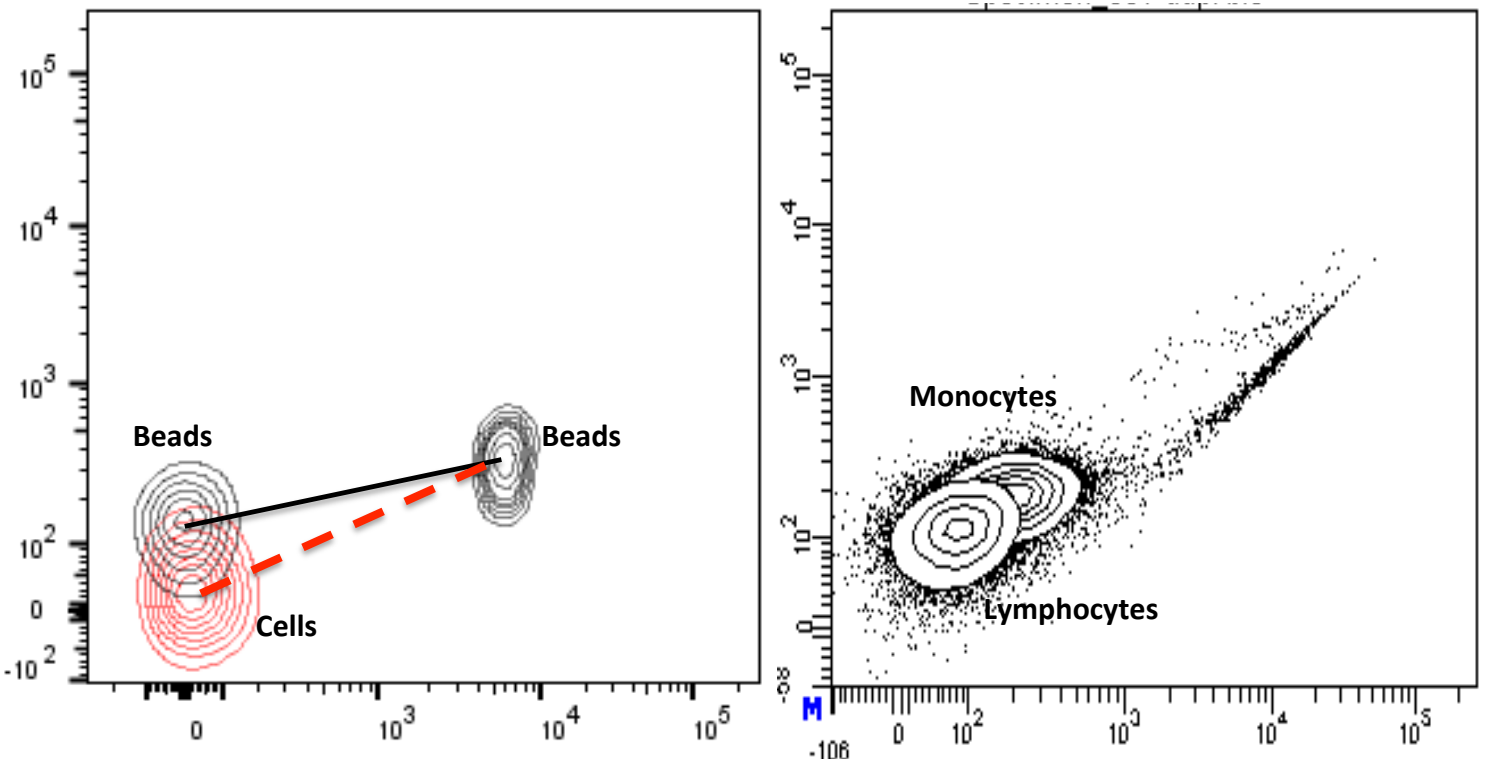
- cells with cells, same subset of cells

  use CD3+ lymphocytes to CD3- lymphocytes.

  don't use CD3+ (lymphocytes) and CD3- (monocytes, granulocytes)

Don't use beads and cells to compensate a single fluorochrome

Rule # 3: Same Autofluorescence



The slope of the line used to calculate compensation is different, depending on which negative autofluorescent population is used.

A mismatched negative will generate an erroneous compensation correction.

Compensation Controls – Cells

Single color stained cells

Pros:

- Best possible match of spectra
- Works with all fluorochromes

Cons: problematic when

- Not enough cells available
- the antigen expression is not known or dim
- Few cells express antigen

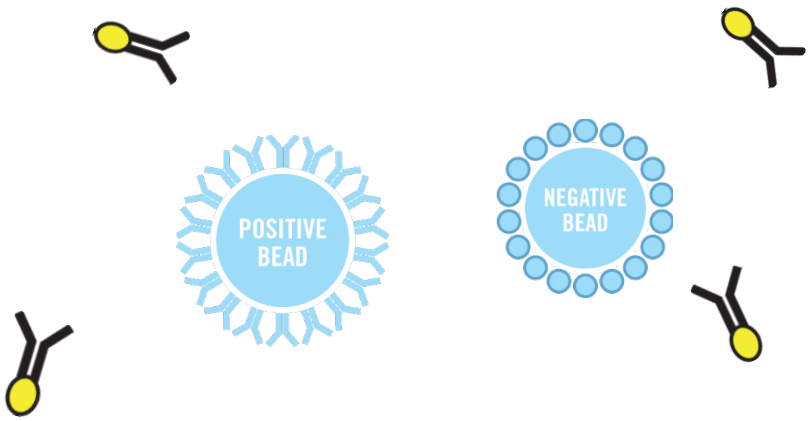
Compensation Controls – Beads

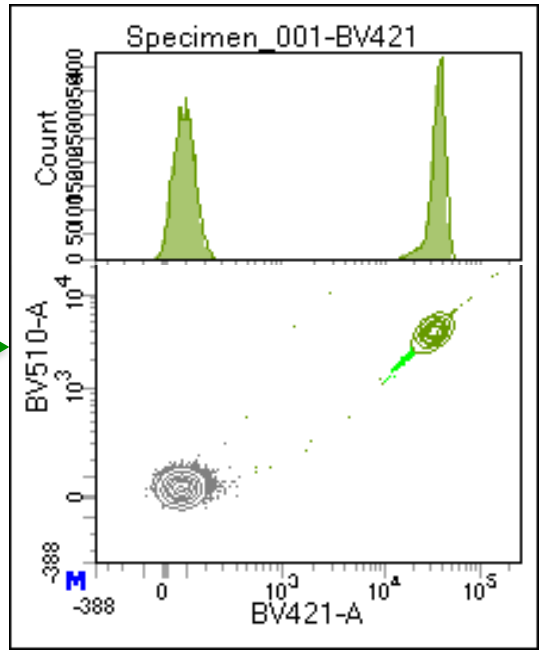
Attach to most antibodies

Give bright positive population

For most fluorochromes give the same SOV as cells

Use beads in same conditions as cells, fixation, detergent





Compensation beads

- Several kinds on the market from different companies
- Available for various situations
  - Live dead amine dyes
  - Compbeads plus: to match high autofluorescence
  - Low autofluorescence beads for violet laser
  - GFP and Cherry beads

Considerations When Using Compensation Beads

Beads are a surrogate for cells, They not a perfect match.

There can be minor differences in spillover

Compensation beads do not provide accurate spillover values when used with certain fluorochromes.

This is often, but not always stated in the antibody product sheet

For example V500 and BUV737

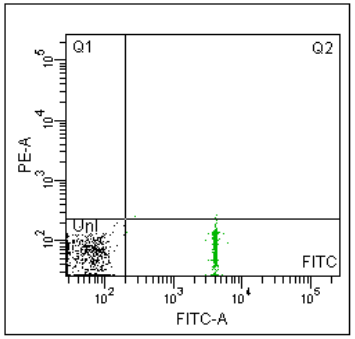
	BUV737 Spillover into					
HuCD4	**BUV395**	**BV711**	**BV786**	**APC**	**AF700**	**APC-H7**
Cells	**2%**	**4%**	**3%**	**0%**	**47%**	**12%**
Beads	**2%**	**5%**	**4%**	**1%**	**56%**	**14%**

Caution!

Compensation value is dependent on:

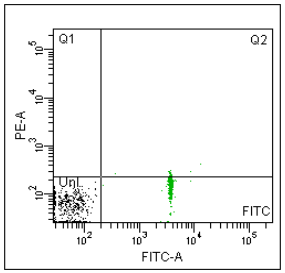
the PMT voltage of the fluorescent parameters

Correct Compensation



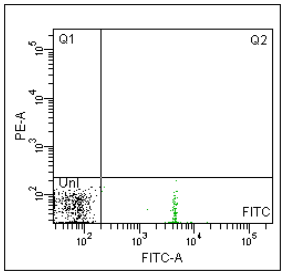
Population	PE-A Mean
Unl	69
FITC	64

PMTV = FITC Decreased by 5 V



Population	PE-A Mean
Unl	
FITC	132

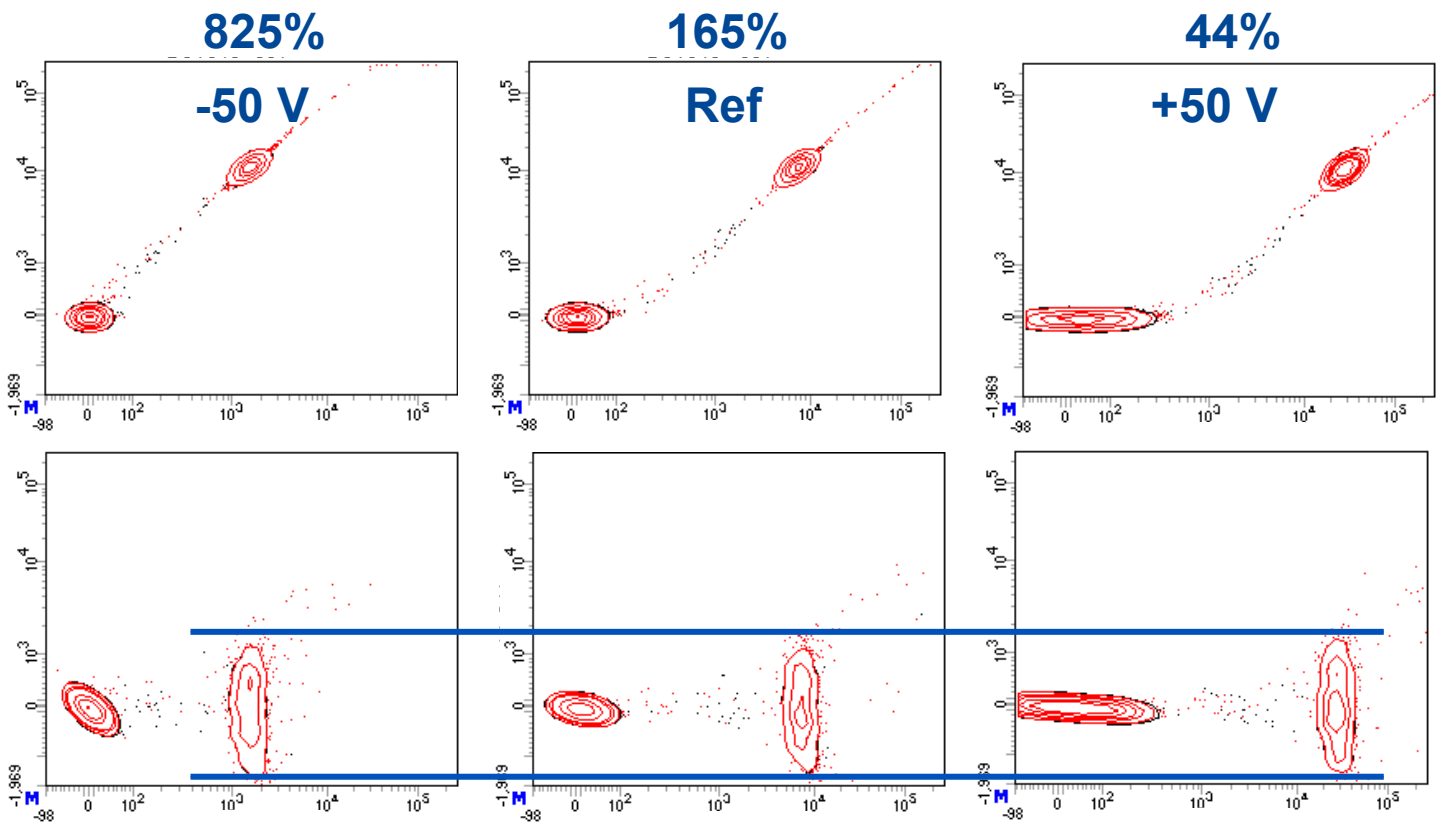
PMTV = FITC Increased by 5 V



Population	PE-A Mean
Unl	67
FITC	49

Never change PMTV for fluorescent parameters after compensation

What is an acceptable Compensation Value?



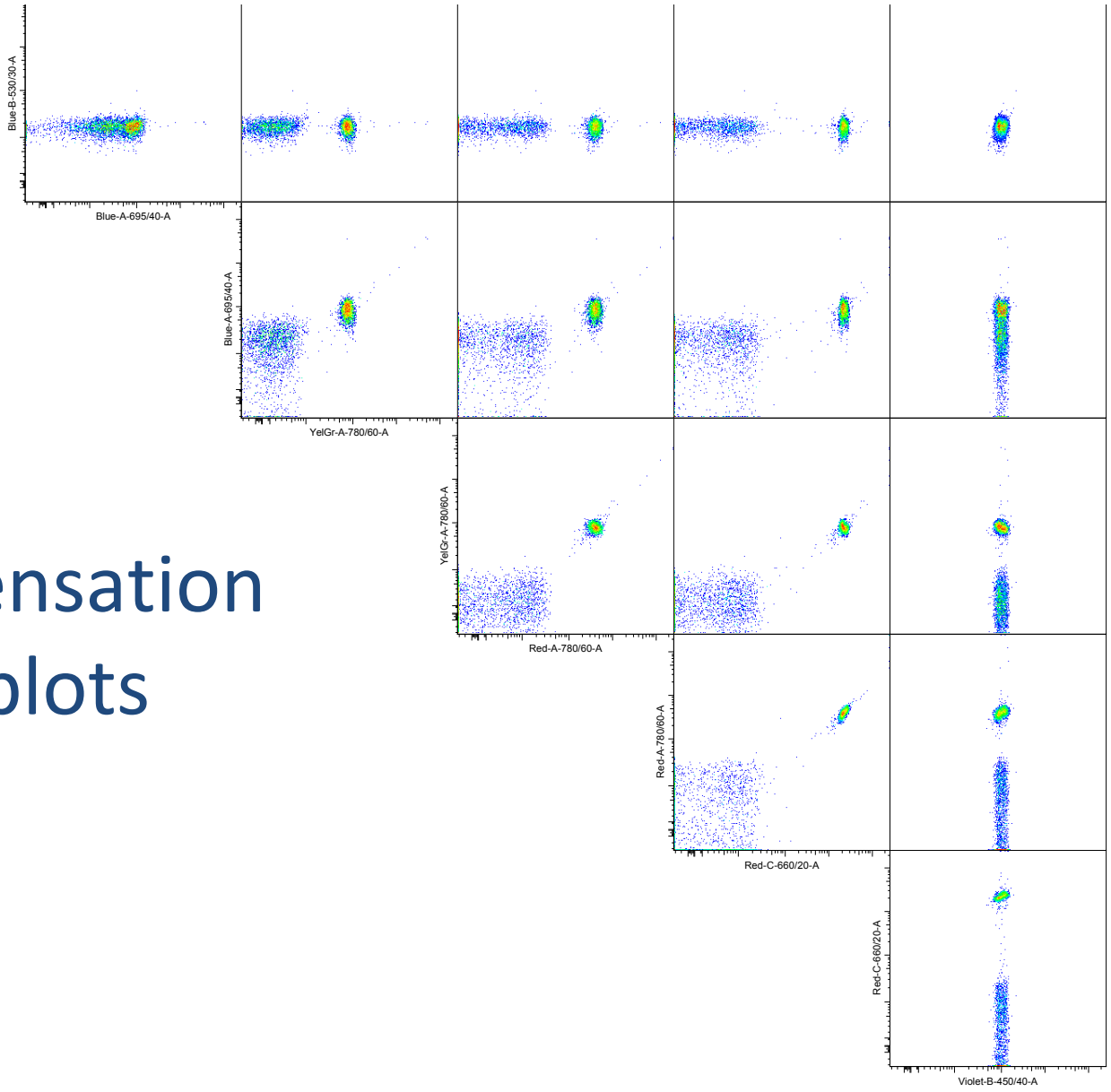
These is the same tube run at 3 different PMT Voltages
The compensation value changes greatly
The result is the same

Check your compensations

- Good compensation dependent on good single colors!
- Look at your single colors with compensation applied
- All colors against all colors
- Make sure they look right, no leaners!
- Correct manually if necessary

  But only on the single colors!
- Then apply corrected values to experimental data

Check compensation with nxn plots

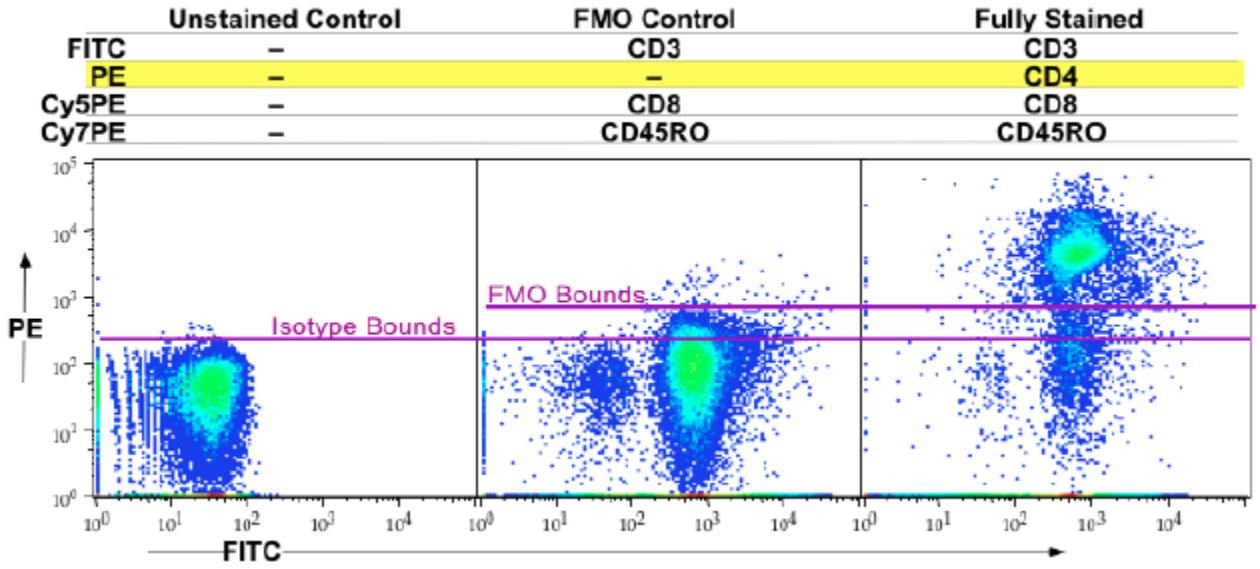


Fluorescent minus one (FMO) controls

- Fluorescence Minus One (FMO) controls contain all the fluorescent markers except one.
- Typically controls for increased background due to spectral spillover related data spread
- For low density or smeared populations (eg, activation markers), FMOs allow accurate delineation of positively vs negatively stained cells

Controls: FMO

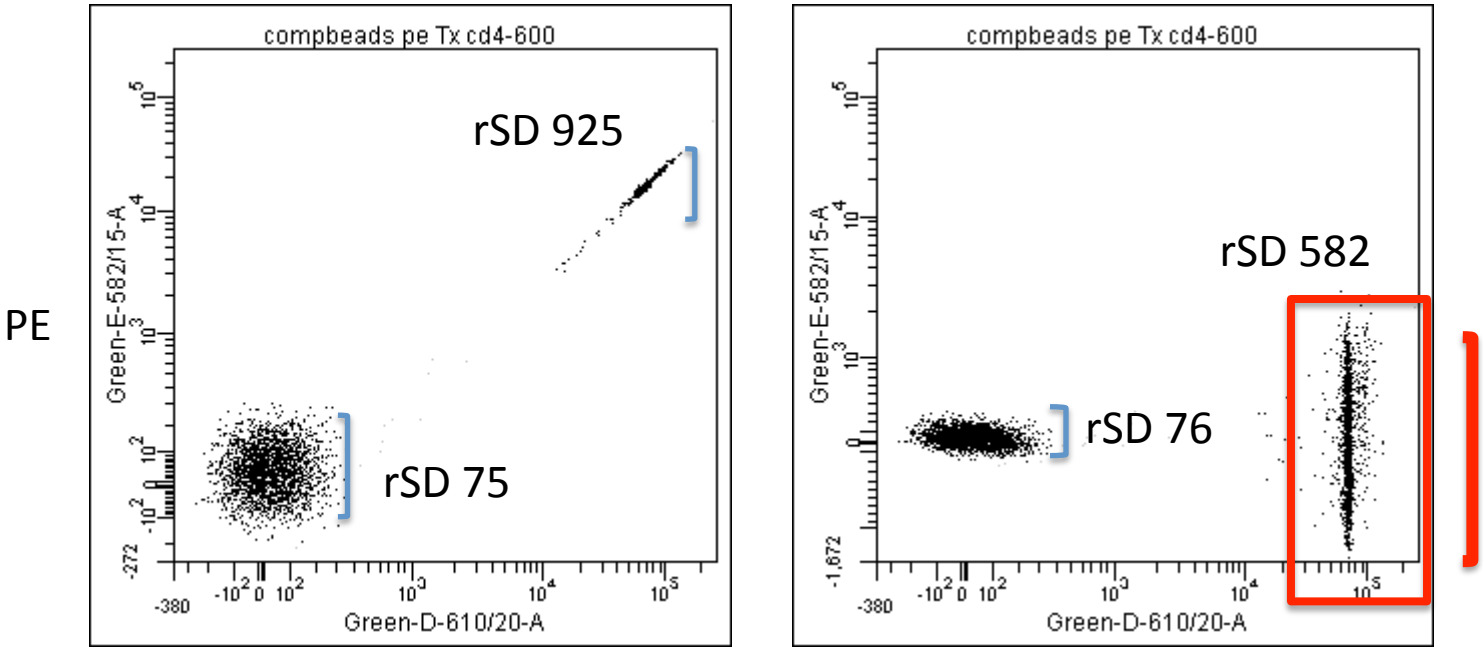
- An FMO (fluorescence minus one) is now considered to be the best control for determining where the threshold for positive cells is.
- An FMO includes all of the fluorochromes in an experiment except for the one of interest which requires a threshold for positivity.
- A good explanation at: http://flowjo.typepad.com/the_daily_dongle/2011/09/fmo-vs-isotype-controls.html



Summary

- Know your cytometer
- Choose your fluorochromes carefully for excitation, emission and brightness
- Look at spectra to determine possible spillover problems
- Good compensation is crucial
- **Good compensation is dependent on good quality controls**
- Gating on positive should be done on FMOs for difficult antigens

Good news:
You can correct for spectral spillover by compensation



PE

PE-TxR

Bad news:
You cannot eliminate ‘spread’ in the positive population

Reference:

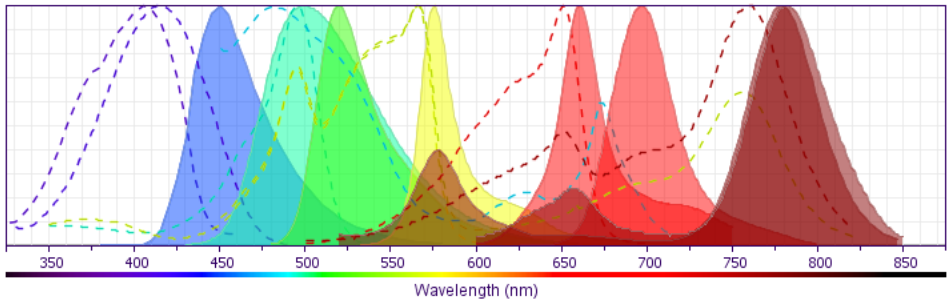
Maciorowski, Z., Chattopadhyay, P.K., & Jain, P. (2017). Basic multicolor flow cytometry. Current Protocols in Immunology, 117, 5.4.1–5.4.38. doi: 10.1002/cpim.26

Multicolor Panel Design

Zosia Maciorowski

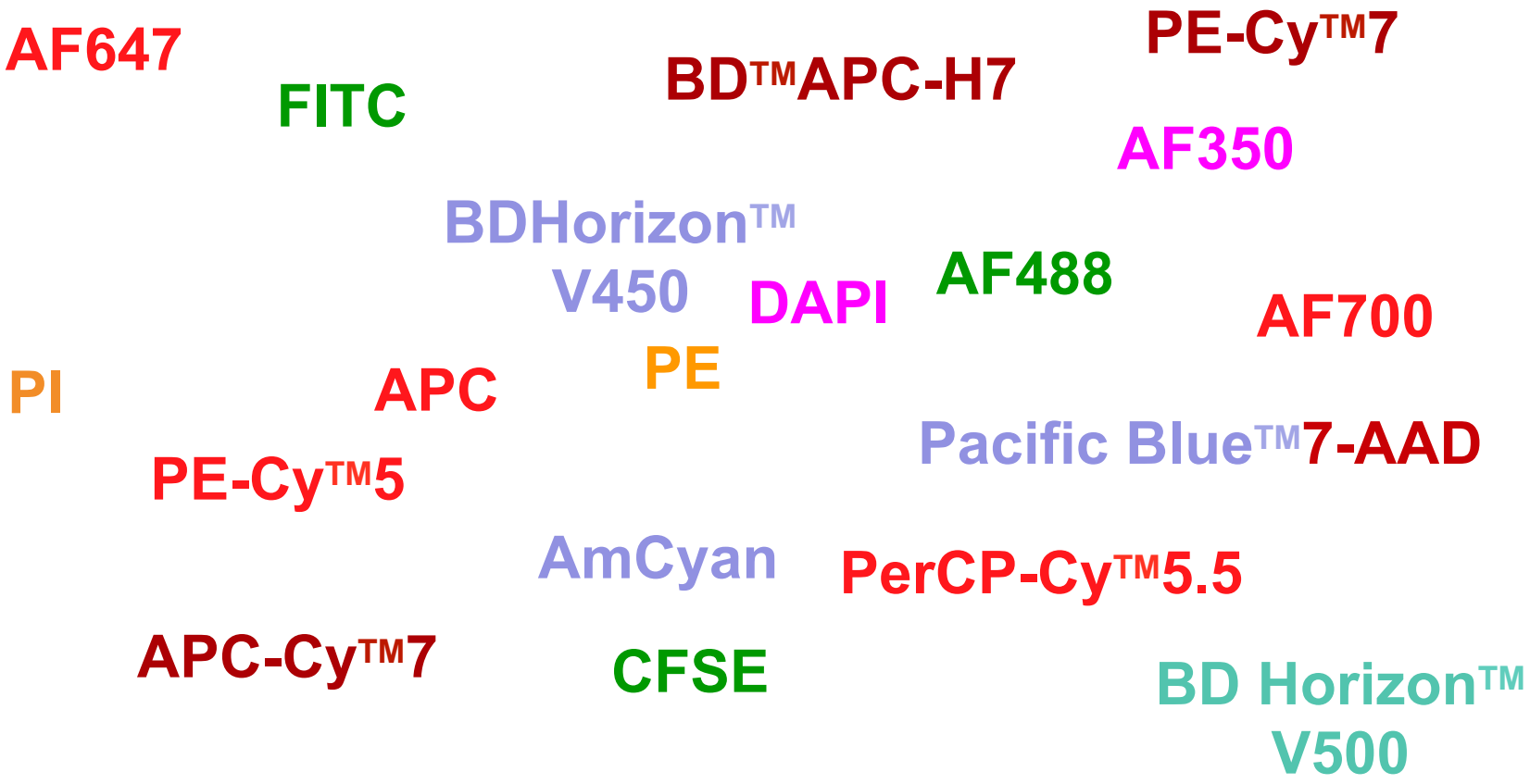
Institut Curie

Paris, France

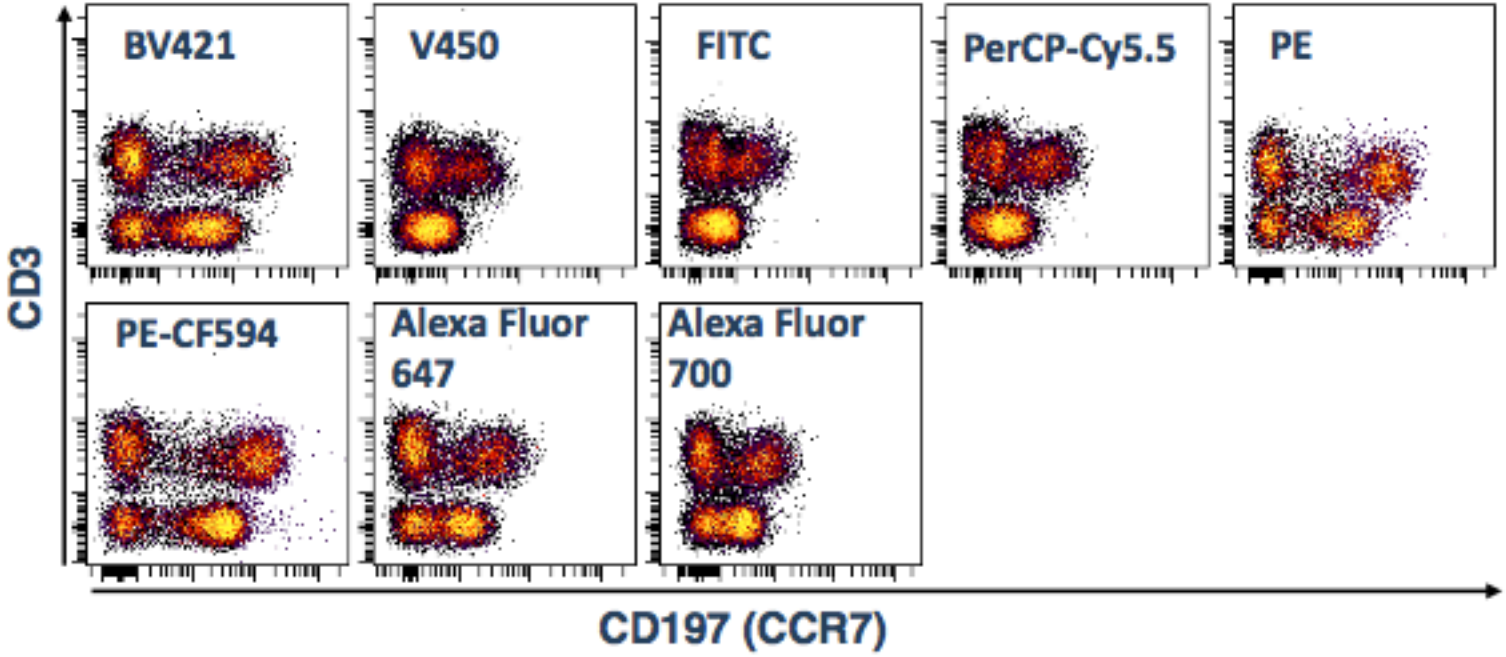


DESIGNING A MULTICOLOR PANEL: Fluorochromes Galore!

But how to combine them for multicolor experiments?



Fluorochrome Choice is Key



- Choosing the correct combination of fluorochromes is key to answer biological questions

BD

How to choose fluorochromes

1. Know your cytometer: lasers and filters, what is possible

2. Know your antigens: priority, expression and density

3. Match fluorochrome brightness with antigen density

   low density antigens need bright fluorochromes
   high density antigens are ok with dim fluorochromes

4. Look at coexpression of 2 or more antigens on same cell

5. If there is coexpression:

   choose fluorochromes very carefully!
   avoid high spread situation from a high expressor into a low expressor.

Cytometer Configuration

Your fluorochrome choice will be determined by your cytometer

- Know what colors you can use before you start

You may have a choice of different cytometers

- Lots of lasers are good even if you don't need all the colors
- High laser power is good; you will get better resolution of weak staining
- Often filters can be easily changed to improve your sensitivity

Know your cytometers

- Characterize stain index and SSM (your core facility may have done this)
- These will be different from machine to machine

If you're planning on sorting these cells in the future, remember stream in air sorters often have lower sensitivity

.

Antigens: Priority and classification

1. Prioritize your markers:

   Which ones are necessary

   Which ones are luxury

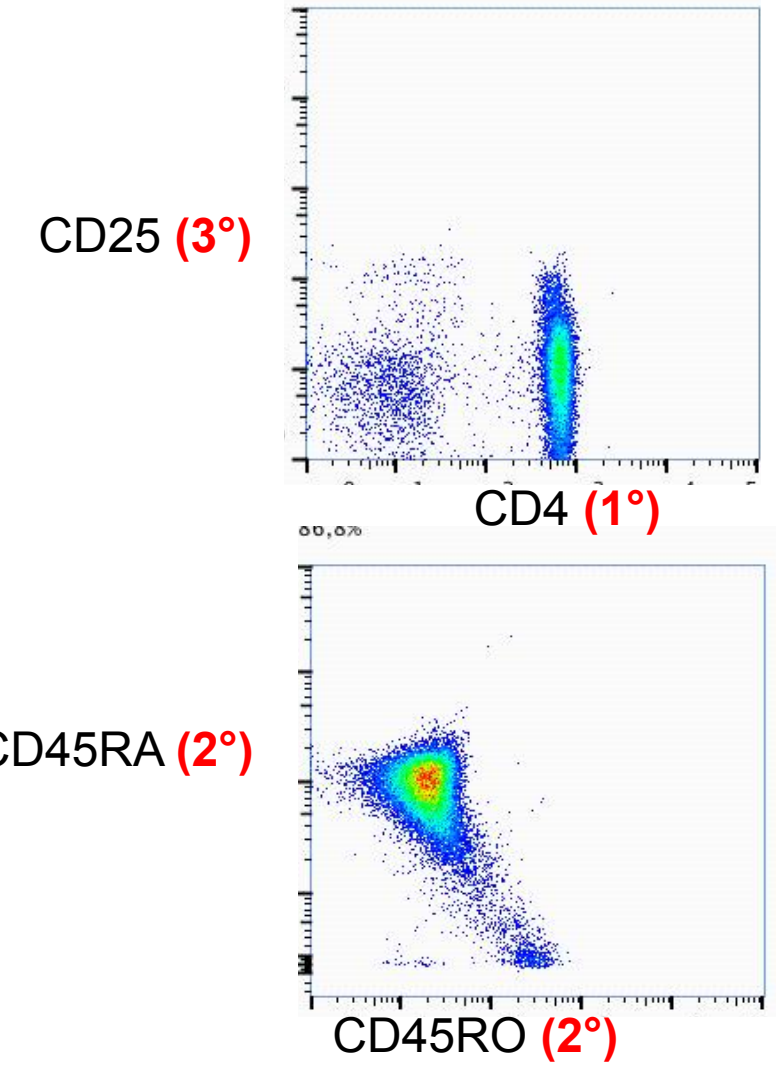
2. Classify your antigens

   1°high density, on or off

   2°high density, continuum staining

   3°low or unknown density

   .

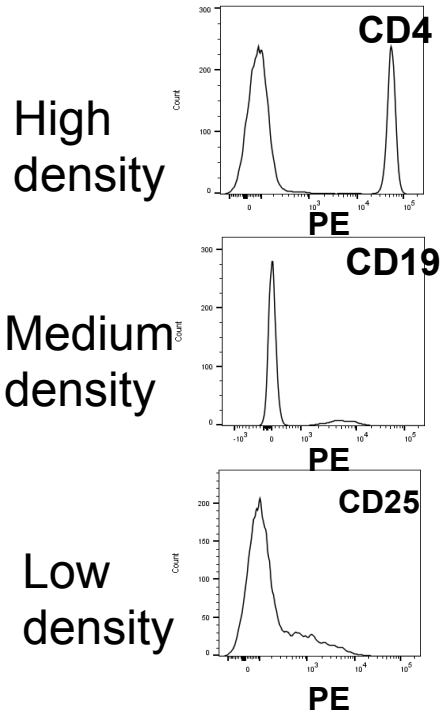


What is your Antigen Density?

Find literature data for density of antigen molecules per cell in **your** system
Low expression antigens will be most difficult to detect

Antigen	Antigen-Density	ExpressionLevel
CD3	90.000	++
CD4	100.000	++
CD8	124.000	+++
CD14	110.000	++
CD19	18.000	+
CD45	200.000	+++
CD56	10.000	+
CD80	2.000	+

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



List of Antigen Densities

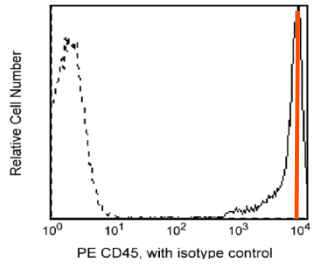
A list of antigen density for 300 antigens is being elucidated by BD and will be published in the near future

Subset	Antigen	Density
Lymphocytes	CD3	32,000
	CD4	36,400
	CD8	65,500
	CD19	7,800
T Cells (CD3⁺CD4⁺ Lymphocytes)	CD25	600
	CD25^{hi}	3,400
	CD27	10,900
	CD28	7,700
	CD45RA	33,400
	CD45RO	12,600
	CD122	5,300
	CD127	2,000
	CD132	400
	CD194 (CCR4)	2,500
	CD197 (CCR7)	2,000
B Cells (CD19⁺ Lymphocytes)	CD20	24,600
	CD24^{mid}	3,000
	CD24^{hi}	16,100
	CD27	3,200
	CD38^{mid}	2,800
	CD38^{hi}	15,900
	CD138	400
	IgD^{mid}	4,900
	IgD^{hi}	23,800
	IgG	28,100
	IgM	3,800

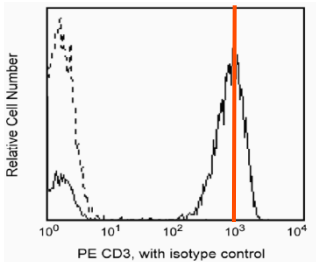
BD

Choose your fluorochromes based on antigen density

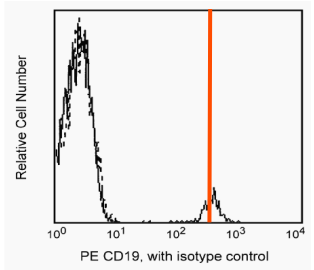
Here we are using PE-coupled antibodies: PE is a very bright fluorochrome



CD45 PE

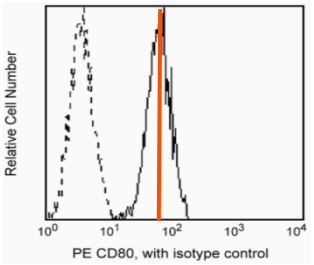


CD3 PE



CD19 PE

CD3, CD45 and CD19 are all high density antigens which don‘t need a bright fluorochrome
Don‘t waste your PE here, use a dimmer fluorochrome.



CD80 PE

CD80 is a low density antigen which needs a bright fluorochrome like PE for good resolution of it‘s dim staining

Pairing Fluorochromes with Antigen Density

SI (Stain Index) is a measure of staining intensity on a specific cytometer

Fluorochrome	SI
BD Horizon™ V450	65
BD Horizon™ V500	27
AmCyan	37
Alexa Fluor® 488	68
FITC	43
PE	305
APC	263
Alexa Fluor® 647	184
PE-Cy™5	198
PerCP	30
PerCP-Cy™5.5	99
Alexa Fluor® 700	64
APC-Cy™7	36
BD™ APC-H7	25
PE-Cy™7	122

Use bright fluorochromes to detect weakly expressed antigens

Use intermediate to bright fluorochromes to detect intermediately expressed antigens, or antigens of unknown expression-levels

Use dim fluorochromes to detect highly expressed antigens

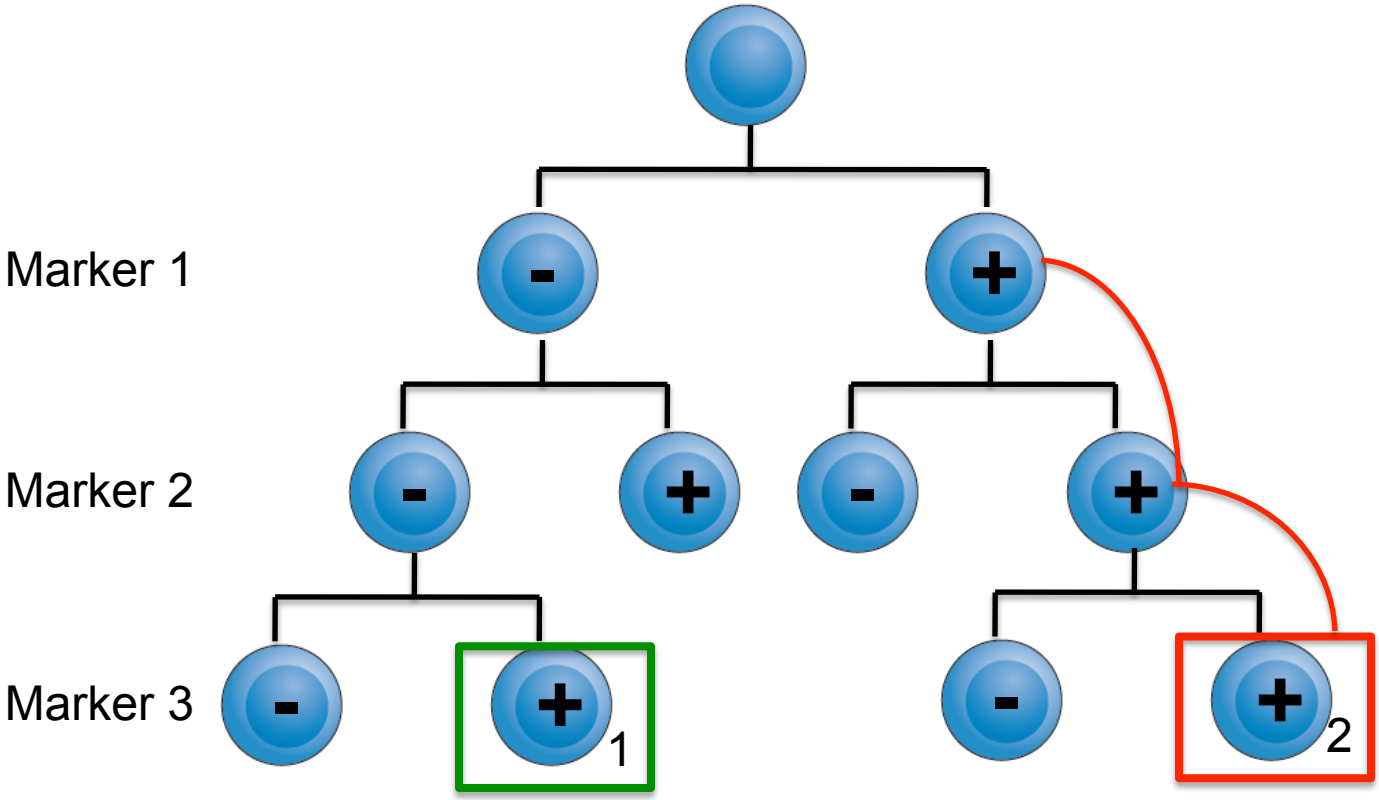
Is there co-expression?

Coexpression of 2 or more antigens on the same cell

can make detection of dim double positives very difficult

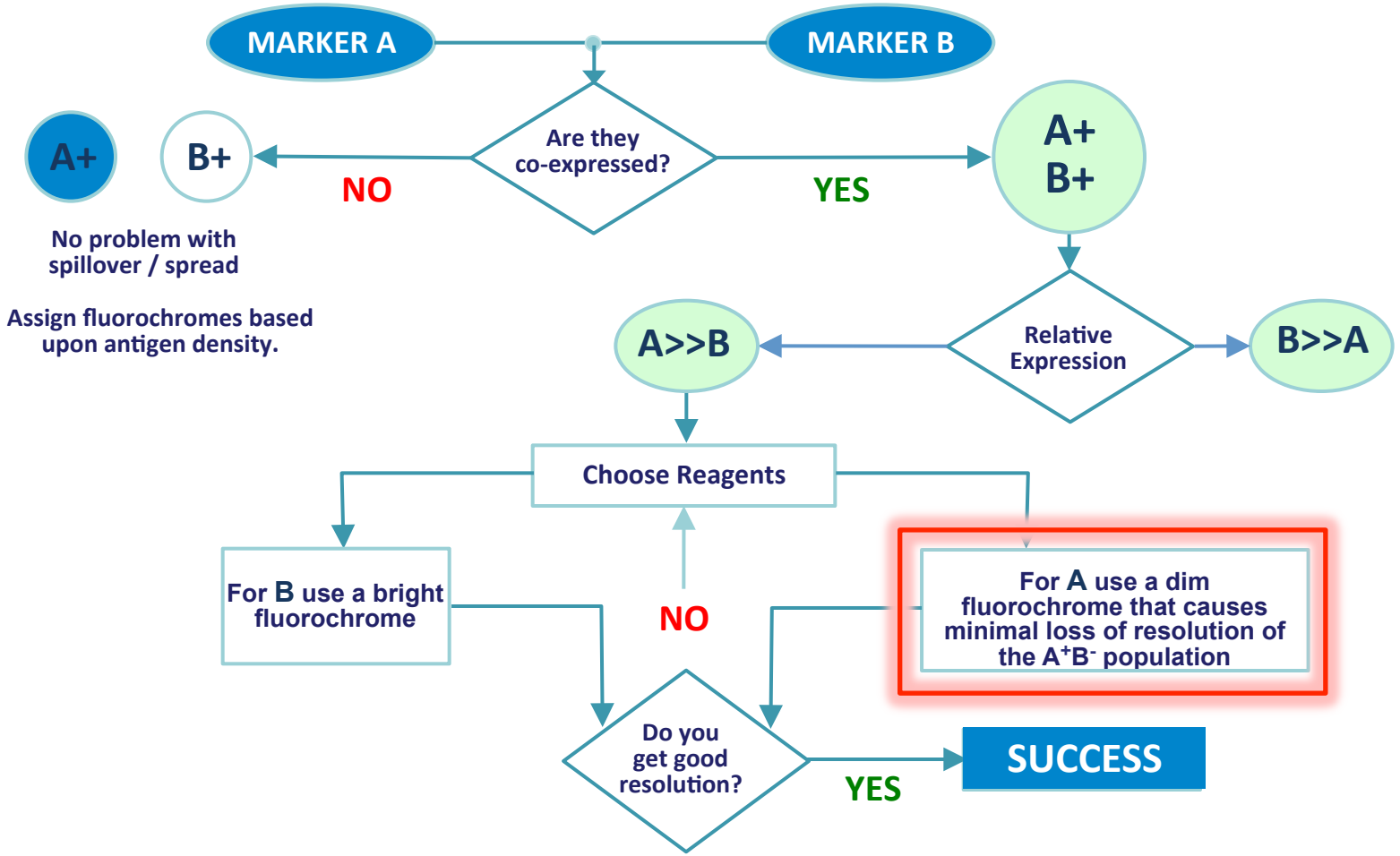
due to spread from multiple fluorochromes

Coexpression



From this experiment strategy, it can be seen that cell 1 is positive only for marker 3, thus has no co-expression. Cell 2 coexpresses all of the other markers, so care must be taken in fluorochrome choice to ensure resolution if some of these markers are dim.

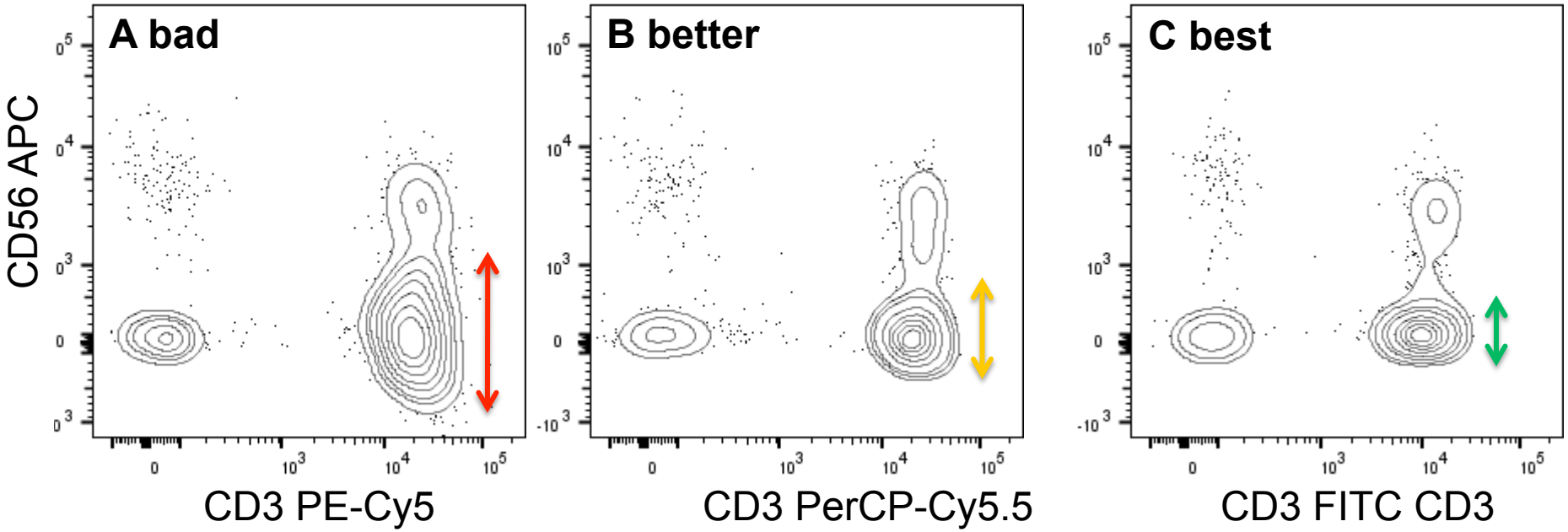
How to Manage Coexpression and Spread



Co-expression and spread
choose your fluorochrome pair carefully

s

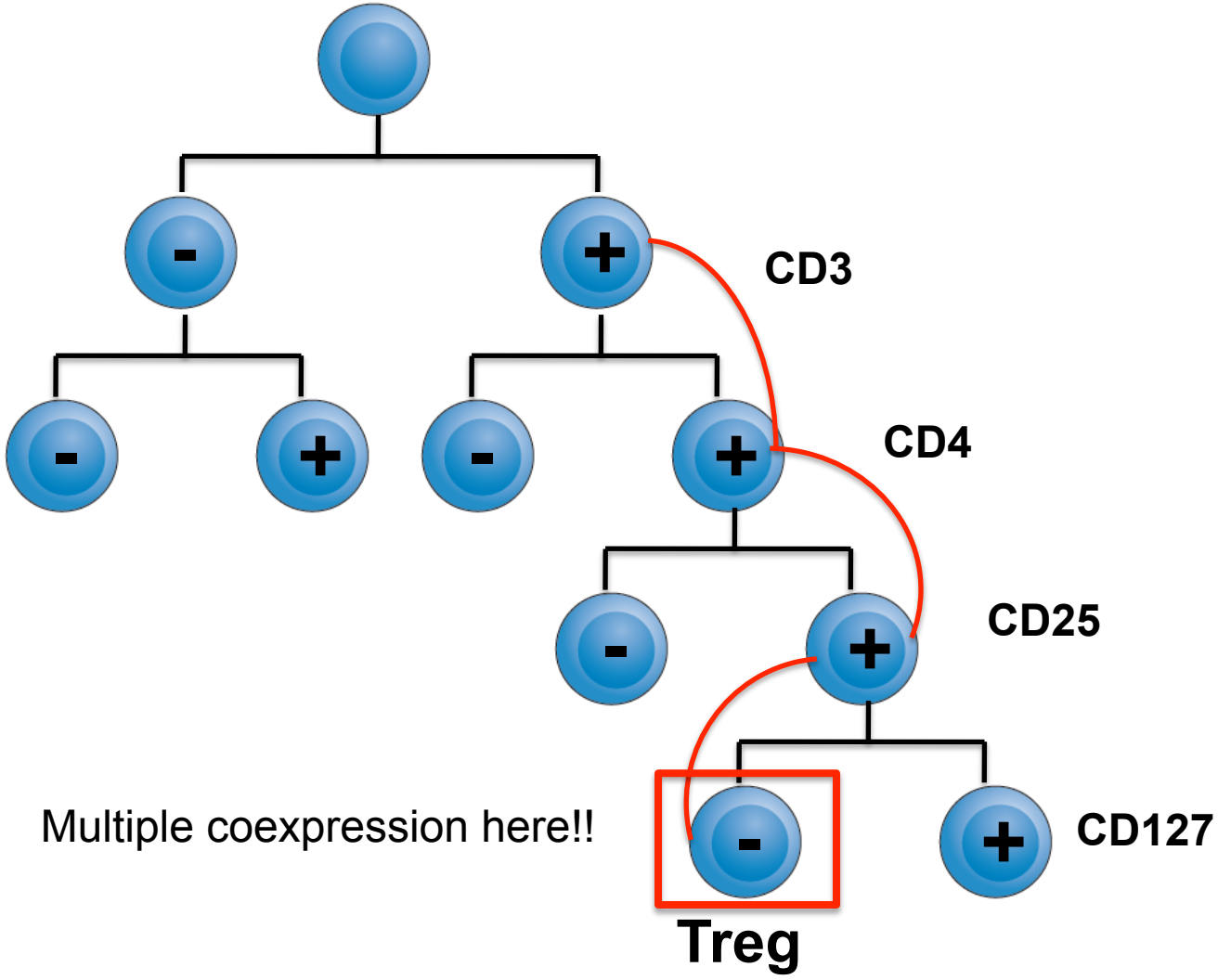
CD56-APC, a low expressor, is shown here paired with 3 different CD3-coupled fluorochromes.



A. The high spread of PE-Cy5 into APC here prevents resolution of the CD56+CD3+ cells
B. Less spread with CD3-PerCP-Cy5.5: resolution is better
C. Very little spread with CD3-FITC allows good double positive resolution. FITC is not bright, but certainly adequate for a high expressor like CD3

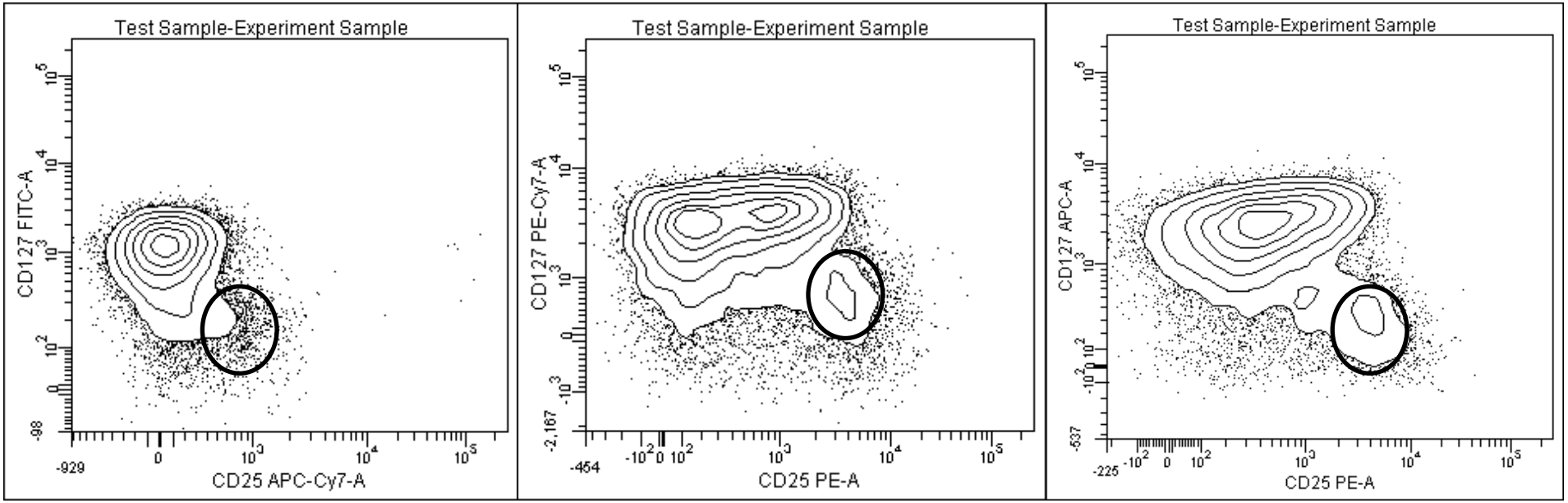
Rule: Choose a dim fluorochrome for high expressor (CD3) which doesn't spread into low expressor (CD56) channel. Choose a bright fluorochrome for low expressor.

Coexpression on Tregs: the real world



3 different fluorochrome pairs for Tregs

CD127 and CD25 are both low density antigens



Problem:
FITC and APC-Cy7 don't have spillover spread but they are **dim** fluorochromes

Problem:
PE and PE-Cy7 are bright fluorochromes, but spillover spread from PE-Cy7 into PE diminishes resolution

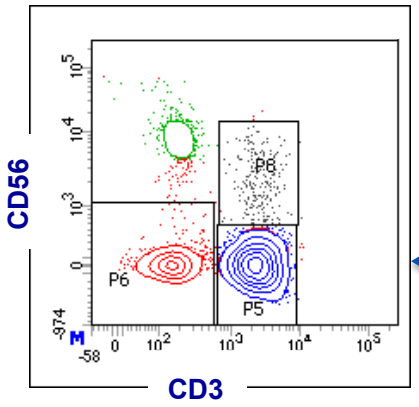
PE and APC are bright fluorochromes with little spread.

Strategies to deal with spread

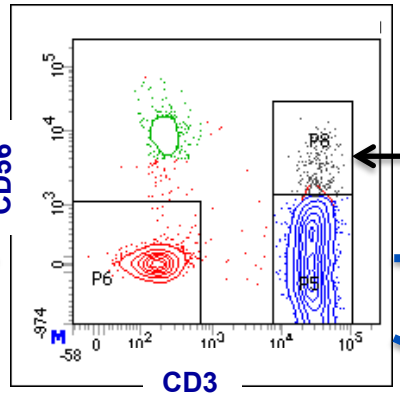
- Use a brighter fluorochrome on the weak antigen
- Use a fluorochromes that don’t overlap
- Dim the staining on the bright coexpressed antigen by diluting the conjugated antibody

How To Minimize the Impact of Fluorescence Spillover to Maximize Resolution

$$\text{Resolution} = \text{SI} = \frac{\text{Brightness}_{\text{MFI}}}{\text{Width of Negative}_{\text{SD}}}$$

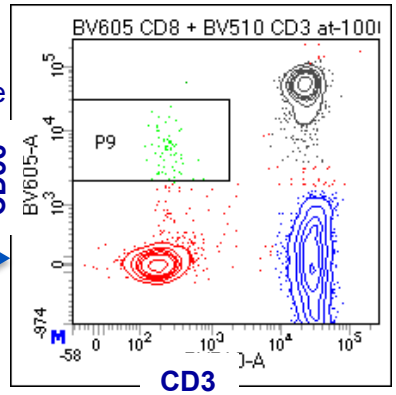


Dilute the CD3 with unconjugated antibody to reduce the spread.



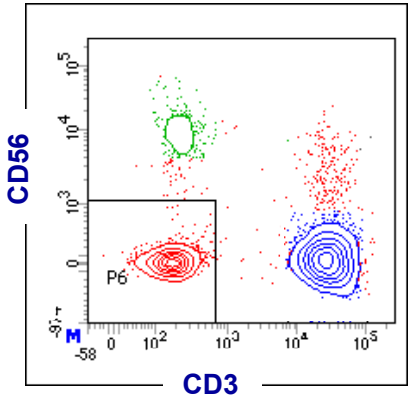
Use a brighter fluor for CD56

How can we improve positive population ?



Understanding the impact of fluorescence spillover on spread is the key to good panel design.

Use a fluor for CD3 with less spillover into the CD56 detector.



Hints to conserve channels

Use a dump channel to remove unwanted cells:

- In a single channel you can:
  - use several antibodies coupled to the same fluorochrome against multiple markers to eliminate unwanted positive cells.
- Use to gate out positive cells, Only the negs are of interest
- Can use a moderately bright fluorochrome with high spread into other channels

Include a dead cell marker

- Helps eliminate false positives and background

Can combine dump and dead cell channel: PerCP5.5/7AAD, BV510/FVS510

If possible, allow for future expansion:

- plan panel to leave open some channels that are good for dim markers: ideally a high intensity fluorochromes with low spread APC, BV421, BUV395, FITC (limited spillover) for example

Other Problems: Background and False Positives

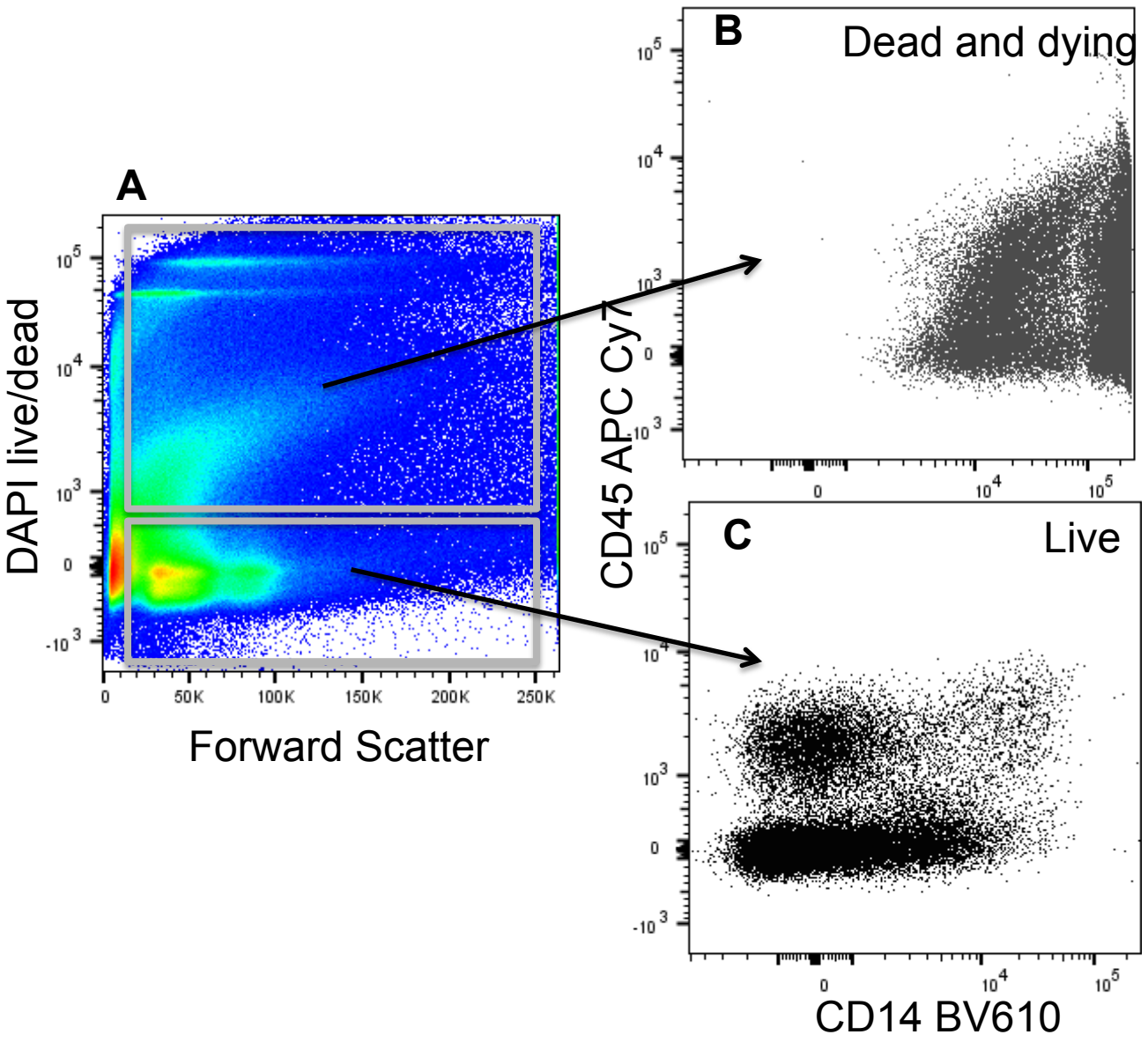
Background:

- Dead cell binding
- High concentrations of reagents (titrate!)
- Non-specific binding of intracellular matrix
- Autofluorescence: varies between populations
  - match red-laser excited fluorochromes with antigens expressed on highly auto-fluorescent cells

False positives:

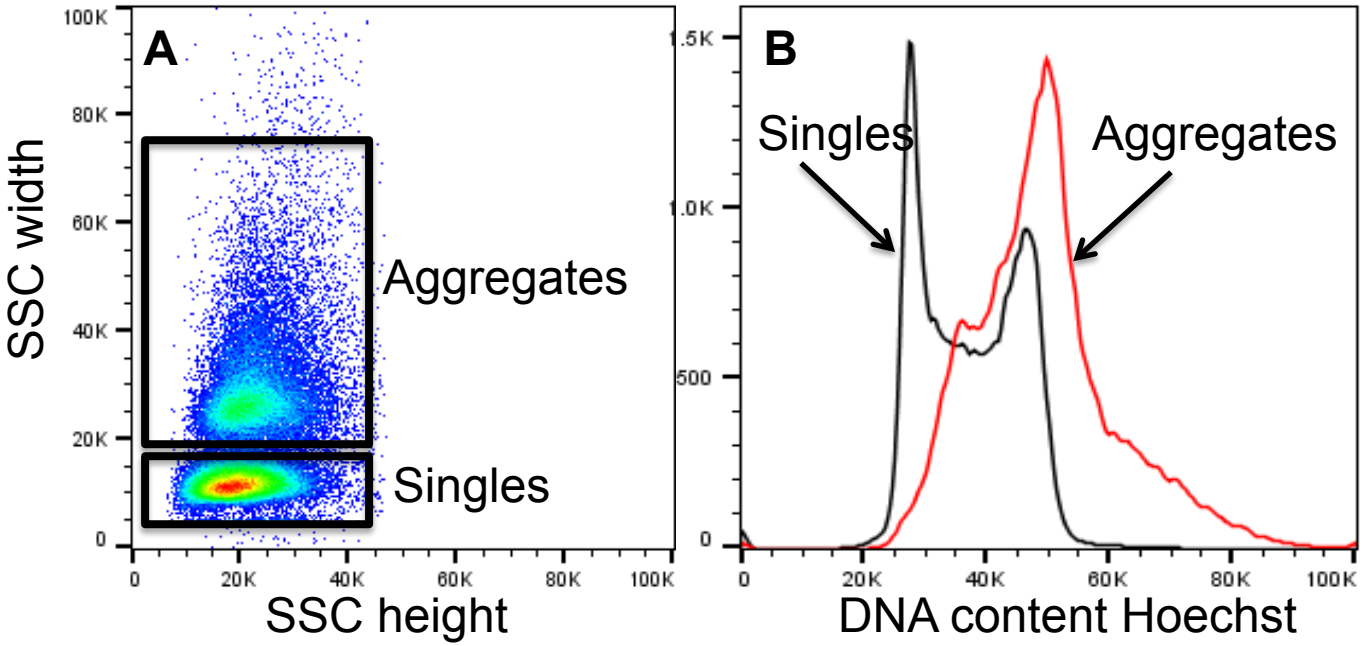
- Aggregates: use doublet discrimination
- Fc receptor binding: use a block
- Beware of Cy dyes (PE-Cy5, PE-Cy7) binding to monocytes and macrophages

Dead cells show as false positive



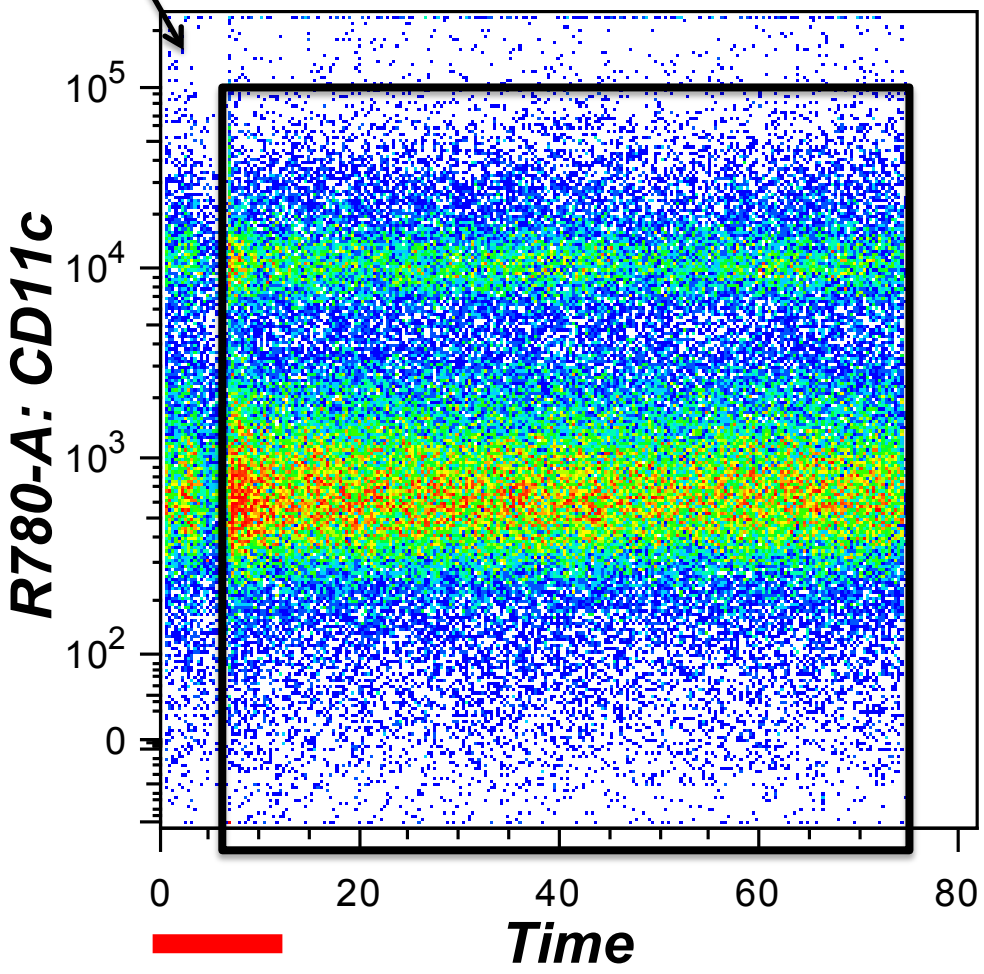
Use Pulse shape to Eliminate Aggregates

Aggregates can appear as false positives



Use a time gate to eliminate artifacts

Fluidics problems during acquisition cause artifacts in the data

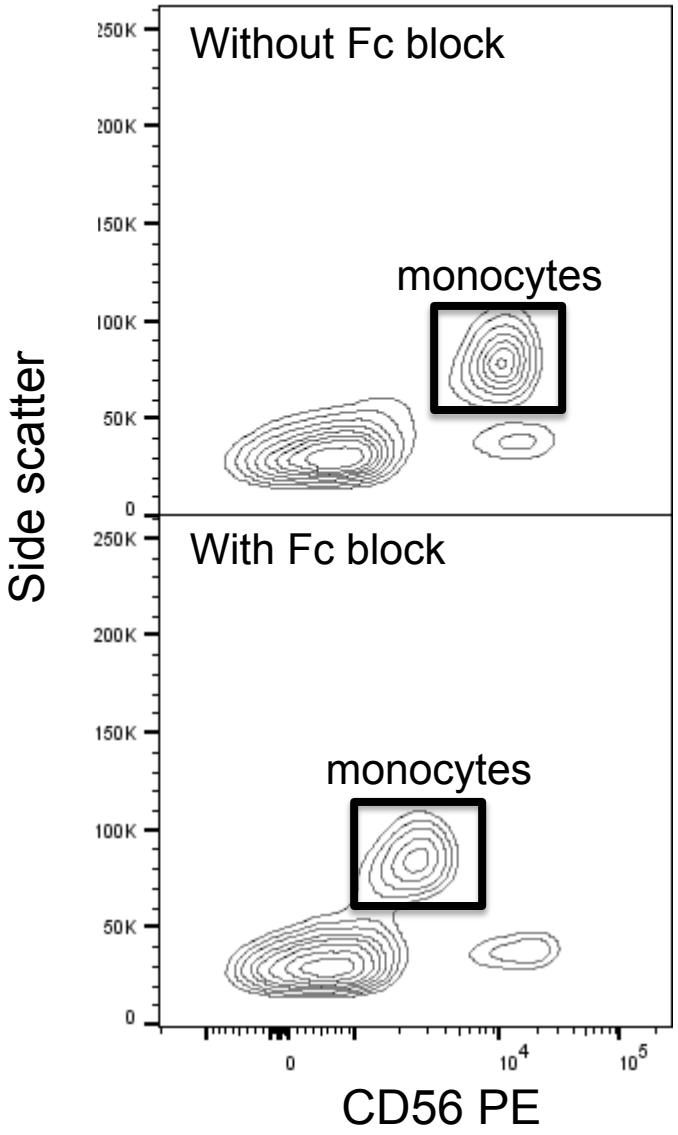


To visualize:

look at data vs time

Then gate out the bad data

Block false positive staining via Fc binding



How to start:Look at your antibody choices

Verify which colors you can run on your cytometer
look at stain index and SSM for your cytometer

Make a table of your possibilities: antigens vs fluorochromes
Often you can ask the manufacturers for samples to test

For rare antigens you may not have much choice of colors
sometimes only fitc or pe
you will have to slot those in first

For 3°antigens you only want to look at bright fluorochromes

Screen the potential antibodies on your cells can to see which ones look best

Make a table of your possibilities

	DR	CD15	CD19	CD123	CD117	CD38	CD34	CD71	CD45	X
PB / V450	●	●	●						●	
FITC	●	●	●	●		●	●	●	●	
PE	●	●	●	●	●	●	●		●	●
PE-TR	●		●				●		●	
P-X	●	●	●	●	●	●	●	●	●	
PE-Cy7	●		●		●	●	●		●	
A594			●			●				
APC	●	●	●	●	●	●	●	●	●	
APC-A700								●		
APC-X7	●		●						●	

Slide courtesy of Dr. Brent Wood

You need lots of controls when you develop your panel

STAINING MATRIX (PANEL 1)									
Tube no.	**BV 421**	**V500**	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**Alexa 647**	**APC-H7**	
1	-	-	-	-	-	-	-	-	Unstained Control (Cell)
2	CD8	CD4	-	-	-	-	-	CD3	Gating Control (Cell)
3	-	-	-	-	-	-	-	-	Negative Bead Control
4	CD8	-	-	-	-	-	-	-	Compensation Controls Using BD CompBeads (except CD4 Tube for which cells are to be used)
5	-	CD4	-	-	-	-	-	-	
6			CD45RA	-	-	-	-	-	
7			-	CD127	-	-	-	-	
8			-	-	CD45RO	-	-	-	
9			-	-	-	CD25	-	-	
10			-	-	-	-	CD197	-	
11			-	-	-	-	-	CD3	
12	-	CD4	CD45RA	CD127	CD45RO	CD25	CD197	CD3	FMO Controls (Cells)
13	CD8	-	CD45RA	CD127	CD45RO	CD25	CD197	CD3	
14	CD8	CD4	-	CD127	CD45RO	CD25	CD197	CD3	
15	CD8	CD4	CD45RA	-	CD45RO	CD25	CD197	CD3	
16	CD8	CD4	CD45RA	CD127	-	CD25	CD197	CD3	
17	CD8	CD4	CD45RA	CD127	CD45RO	-	CD197	CD3	
18	CD8	CD4	CD45RA	CD127	CD45RO	CD25	-	CD3	
19	CD8	CD4	CD45RA	CD127	CD45RO	CD25	CD197	-	
20	**CD8**	**CD4**	**CD45RA**	**CD127**	**CD45RO**	**CD25**	**CD197**	**CD3**	**Experiment sample**

During panel development:
Run comp controls with both beads and cells, sometimes beads don't give same values
Run FMOs for each color
Once developed you will probably only need 1 or 2 FMOs for difficult markers
During panel development, add antibodies sequentially to identify problems
Compare Stain Index of single stained cells with fully stained cells: should be the same if no problem

Validate your panel

Run many samples

- Under same conditions as to be used
- Positive and negative controls

Slide courtesy of Dr. Brent Wood

Summary

Know your cytometer: lasers and filters

Know your Antigens: priority, expression and density

Match bright fluorochromes with low density antigens and dim fluorochromes with high density antigens

If there is coexpression: avoid high spread from a high density expressor into a low density expressor.

If possible, spread antigens across lasers

Leave room for future expansion:

bright fluorochromes with little spillover, APC, BV421, BUV395

Panel design software

Fluorofinder panel design program

https://fluorofinder.com/

BD Biosciences panel design program

https://www.bdbiosciences.com/sg/paneldesigner/index.jsp

Fluorish panel design program

https://www.fluorish.com/

You can load your cytometer configurations into these programs

FCM diagnostics and functional tests in autoinflammatory diseases – clinical cases

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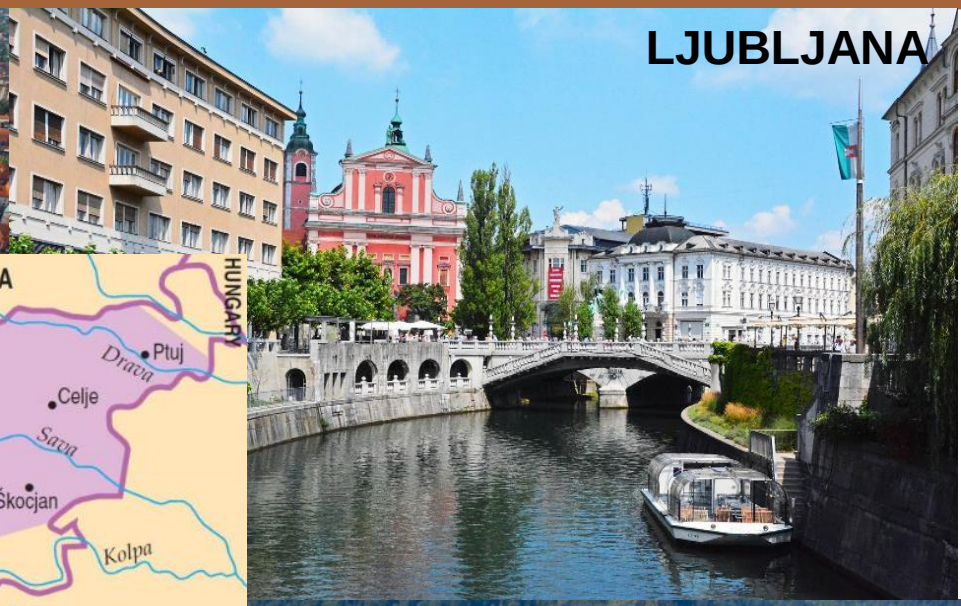
Ljubljana, Slovenia
http://www.imi.si/

Univerza v Ljubljani
Medicinska fakulteta

Welcome to Slovenia & Ljubljana!!

City of Ljubljana



Welcome to University Ljubljana Faculty of Medicine!!

- UL: 40 000 undergraduate and graduate students, 5.600 employees, 23 faculties and three arts academies
- FM: 1500 undergraduate and graduate students, 850 employees

Inštitut za mikrobiologijo in imunologijo (IMI)

More than 500 different types of diagnostic tests, 615 000 samples / yr; 150 employees (36 PhD, 15 MD, 8 prof.)

http://www.imi.si/

Univerza v Ljubljani
Medicinska fakulteta







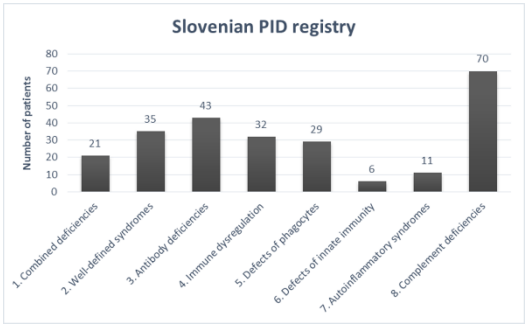


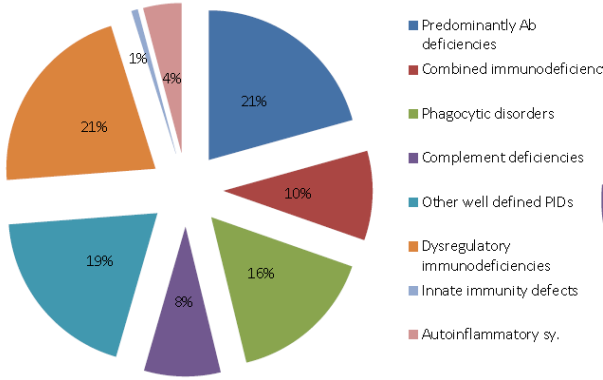


Slovenian PID team

260 registered PID patients, 51 different disease entities, 144 (55%) of Pts according to genetic diagnosis

Department of Allergology, Rheumatology and clinical Immunology, University Children's Hospital Ljubljana





Immunology laboratory – Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana
Medical faculty Ljubljana

Hematopoietic stem cell transplantation team Department of University Children's Hospital Ljubljana

Diagnosis	Age at HSCT
XLP	4 yrs
XLP	3,5 yrs
SCID - OMENN syndrome (RAG1)	3 months
XCGD	5 yrs
XCGD	25 yrs
SCID - Hypomorphic Rag1 deficiency	4 yrs
Osteopetrosis	10 months
APDS (PI3Kδ)	8,5 yrs
AR CGD	21,5 yrs
fHLH	1,5 yrs
SCID - OMENN syndrome	17 months
SCID - JAK3	9 months
XCGD	19,5 yrs
SCID (CD3E) - sibling A	12 months
SCID - OMENN syndrome (RAG1)	5 months
MALT 1 deficiency - sibling A	6,5 yrs
MALT 1 deficiency - sibling B	4,5 yrs
unknown CID, Monosomy 7	5,5 yrs
XCGD	7 yrs
XCGD	3,5 yrs
SCID (CD3E) - sibling B	4 months

Primary immunodeficiencies

Number of PID / 100.000 persons

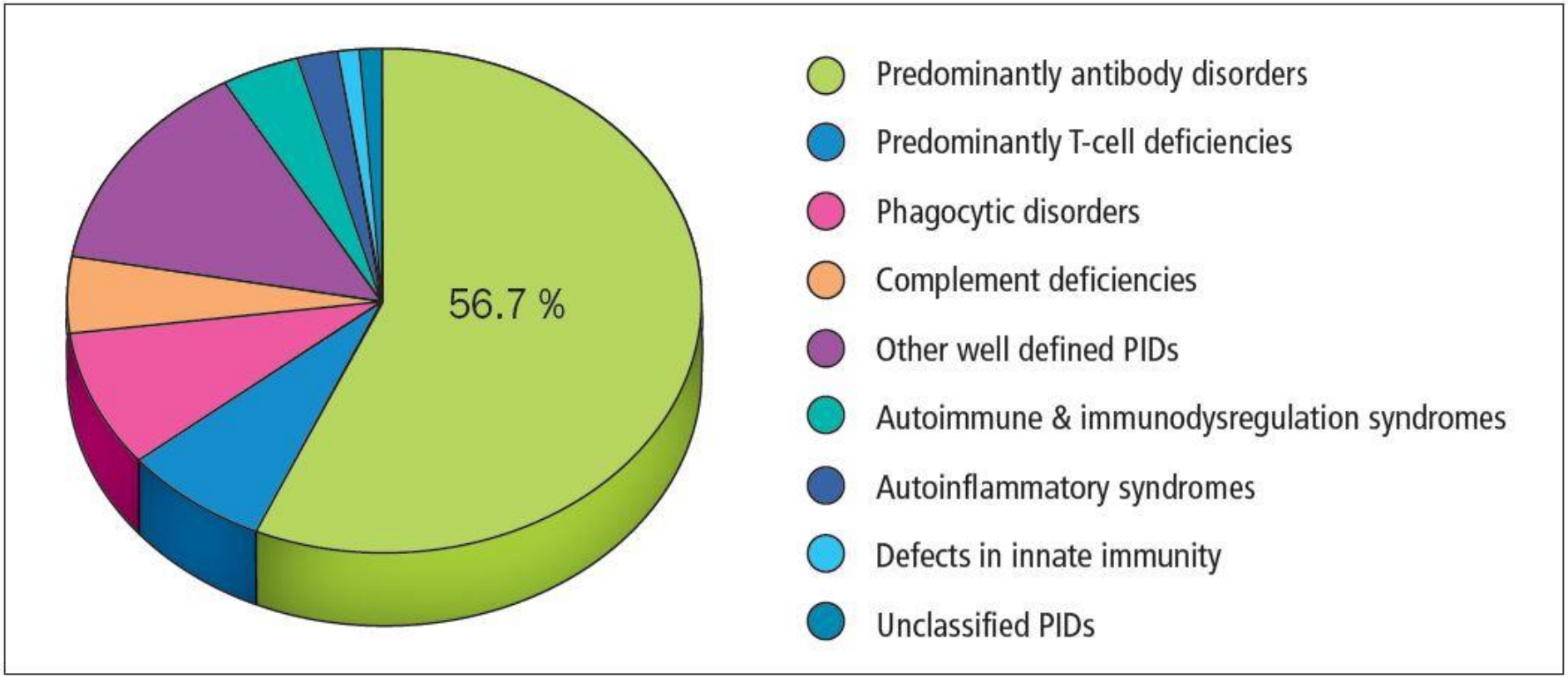
	PID patients	Estimated population	Total PID/population
Prague	454	3.000.000	**15,1**
Vienna	297	2.000.000	**14,9**
Debrecen	564	4.000.000	**14,1**
Ljubljana	260	2.000.000	**13,0**
Tallinn	86	800.000	**10,8**
Bratislava	221	2.500.000	**8,8**
Timisoara	210	2.400.000	**8,8**
Sarajevo	38	500.000	**7,6**
Zagreb	209	3.900.000	**5,4**
Vilnius	51	1.000.000	**5,1**
Kaunas	86	2.000.000	**4,3**
Astana	37	900.000	**4,1**
Belgrade	302	7.500.000	**4,0**
Minsk	380	9.600.000	**4,0**
Sofia	196	7.000.000	**2,8**
Almaty	24	1.500.000	**1,6**
Baku	89	9.500.000	**0,9**
Total	3.504	60.100.000	5,8

- A genetically heterogeneous group of disorders that affect distinct component of the innate and adaptive immune system
- Explosive growth of knowledge

Year	No. of recognized PIDs
1997	60
1999	71
2013	200
2016	> 300

Slide courtesy of T Avčin

Classification of PIDs based on their known molecular causes



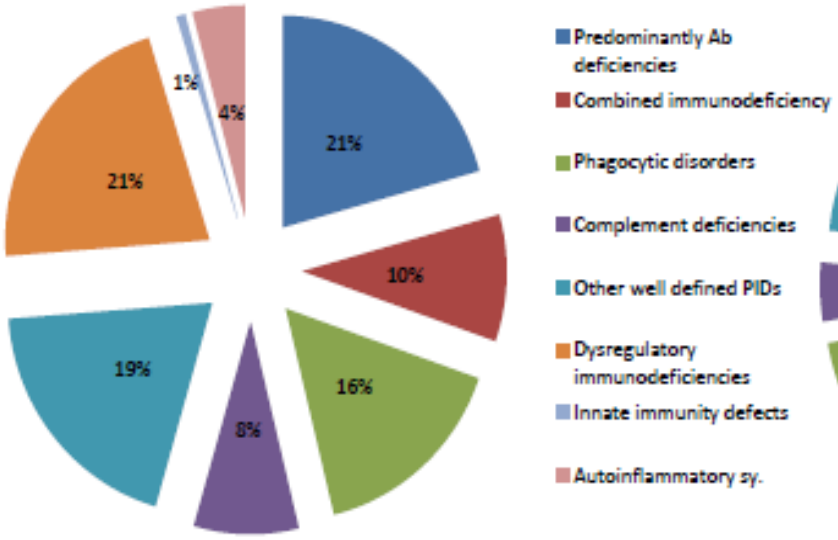
Baumann U. Immunology News 2017;3 - Issue 1, April 2017

Early clinical recognition of PID

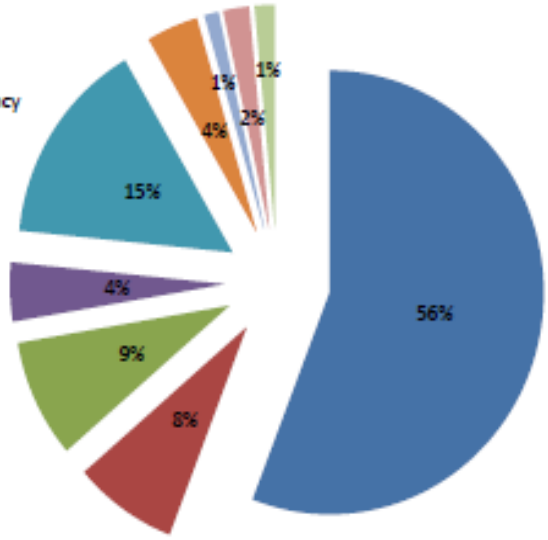
- Impaired antimicrobial host defence
  - More frequent, longer and more severe infections
  - Opportunistic infections

- Impaired surveillance function of immune system
  - Autoimmune manifestations
  - Granulomatosis
  - Hemophagocytic syndrom
  - Lymphoproliferation
  - Solid tumors

Distribution of PID patients in Slovenia

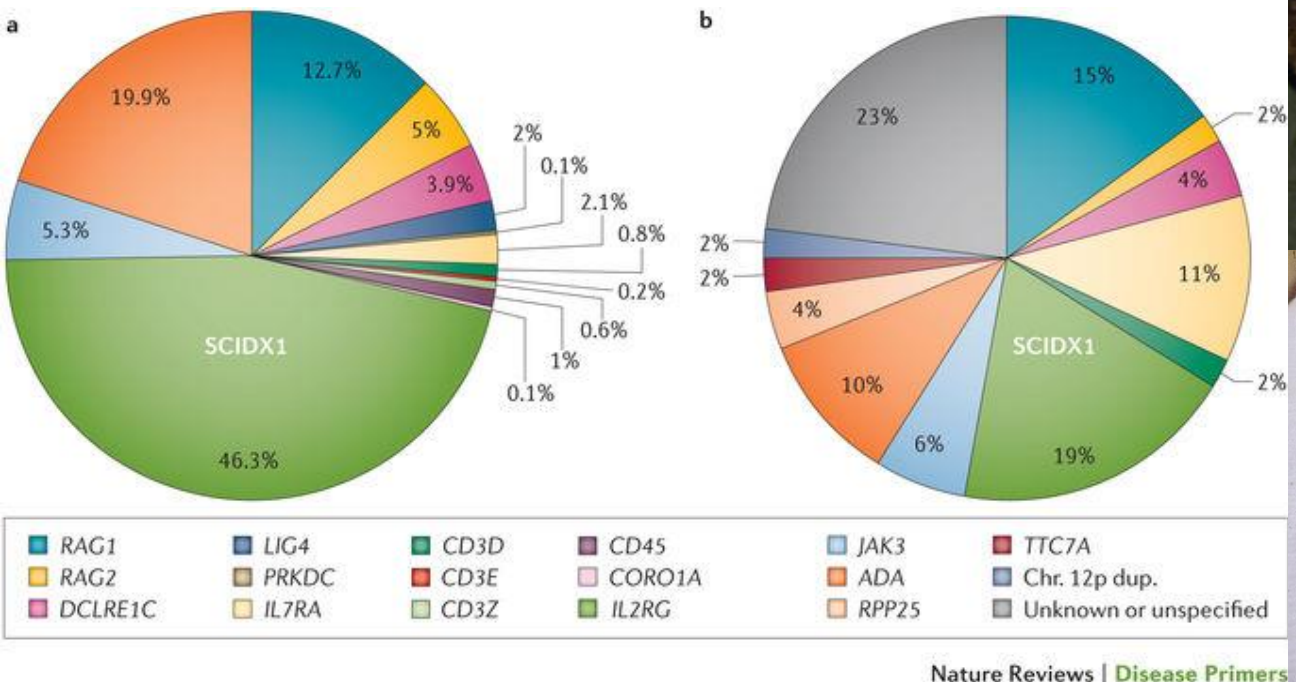


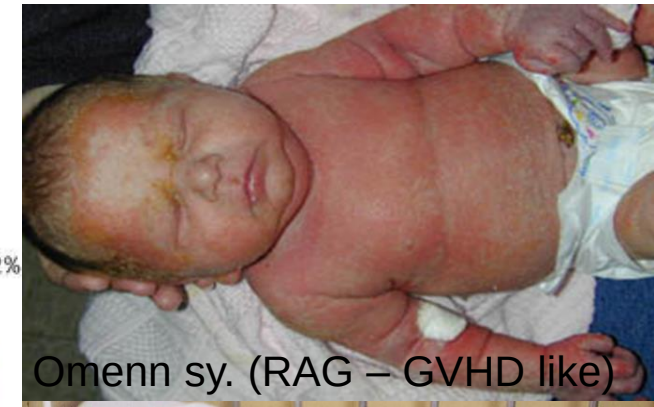
Distribution of PID patients in ESID Registry



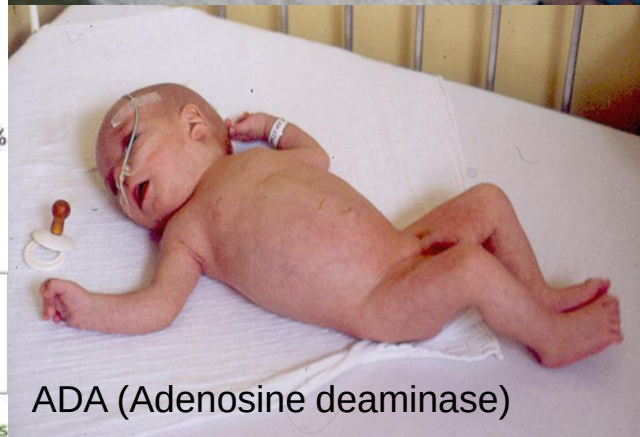
PID with early & serious clinical manifestations: Severe Combined Immunodeficiency (SCID)

- **Genetic defects of T cell development**
  - absence of mature T cells (abrogated adaptive immunity)
  - variably associated with defective differentiation of other hematopoietic cells
- **Considerable genetic heterogeneity**
  - inherited as an X-linked or autosomal recessive disorder
  - at least 22 molecularly defined SCID disorders





Omenn sy. (RAG – GVHD like)



ADA (Adenosine deaminase)

Block in lymphopoiesis caused by SCID

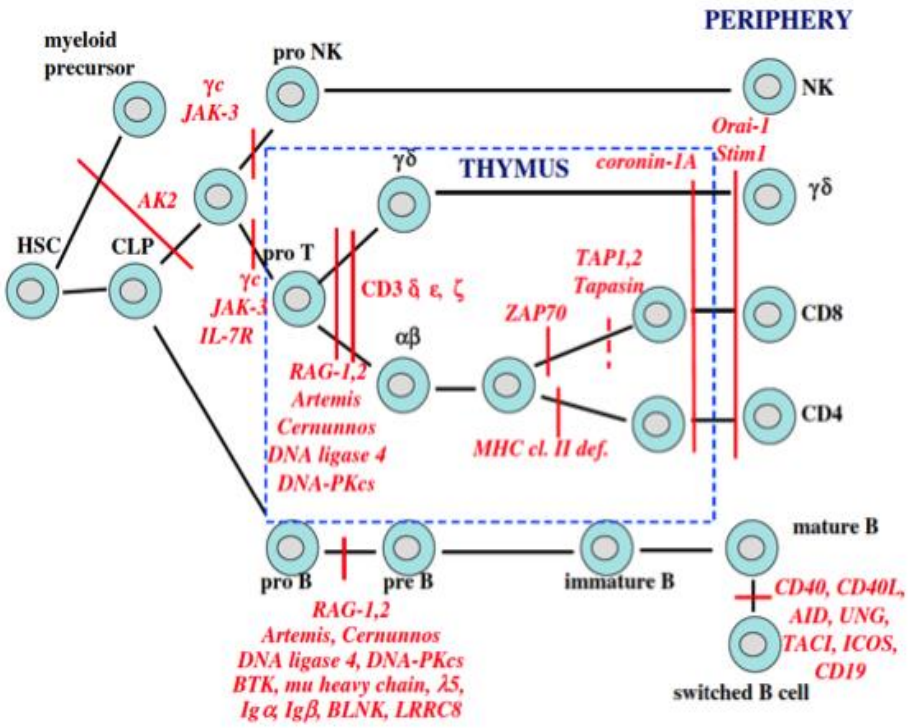


FIG 1. Blocks in T-and B-cell development associated with PIDs.

Notarangelo LD. J Allergy Clin Immunol 2010;125:S182-S194

Defect	Gene Defect	Inheritance	T,B, NK Cells
Cytokine signalling	CgC	XL	- + -
	JAK 3	AR	- + -
	IL7 Ra	AR	- + +
Nucleotide biosynthesis salvage pathway defects	ADA	AR	T_{low} B_{low} NK_{low}
	PNP	AR	$T_{low}B_{low}NK_{low}$
Defects affecting signalling through the T cell antigen receptor	CD45	AR	- + -
	CD3d	AR	- + -
	CD3e	AR	- + -
	CD3z	AR	- + +
	ZAP70 kinase	AR	+ + + (absent CD8)
VDJ recombination defects	RAG 1 & 2	AR	- - +
	Artemis	AR	- - +
	Cernunnos	AR	T_{low} B_{low} NK+
	DNA ligase 4	AR	T_{low} B_{low} NK+
Thymic defects	22q11	Sporadic/AD	T-B+NK+
	CHD7	Sporadic/AD	T-B+NK+
	FOXN1	AR	T-B+NK+
Other	AK2 (RD)	AR	- - - (+ myeloid dysfunction)
	MHC class II deficiency	AR	+++ (absent CD4)
	ORAI1	AR	Ca-dependent T cell activation
	STIM1	AR	

Case 1:

- **First presentation**

  16mo old boy

  1wk subfebrile temperatures

  few days pale, tired, sleepy

  1day small red dots on skin

- **History**

  First child of healthy unrelated Caucasian parents

  Normal development

  Occasional oral thrush from birth

  Few purulent rhinitides since beginning of day care

- **Examination**

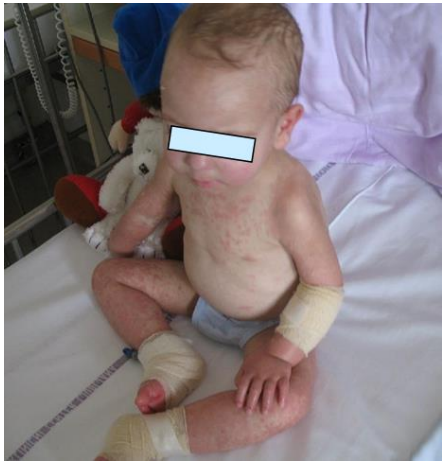
  Pale, afebrile, tachycardia

  Weight between 10-25p

  Petechiae

  Hepatosplenomegaly

  Generalised lymphadenopathy



- **Diagnostics:**

	sep.07
SR	120
CRP	9
L	12,0
Hb	63
Tr	14

Diff.: Neu 24%, Ly 29%, Mo 8%, Eo 29%

Ery 2,54, MCV 76, high Rtc

Lymphocyte phenotyping

CD3+	3350 (* 10^6/l) (1400 - 3600)	54%
CD4+	496 ↓ (700 - 2000)	8%
CD8+	2670 ↑ (500 - 1400)	43%
CD19+	806 (400 – 1500)	13%
CD56/16+	1860 ↑ (100 – 700)	30%
HLA -DR	1550 ↑ (100 – 400)	25%
DNT (CD3+ CD4-CD8- TCRαβ+)		5,8%

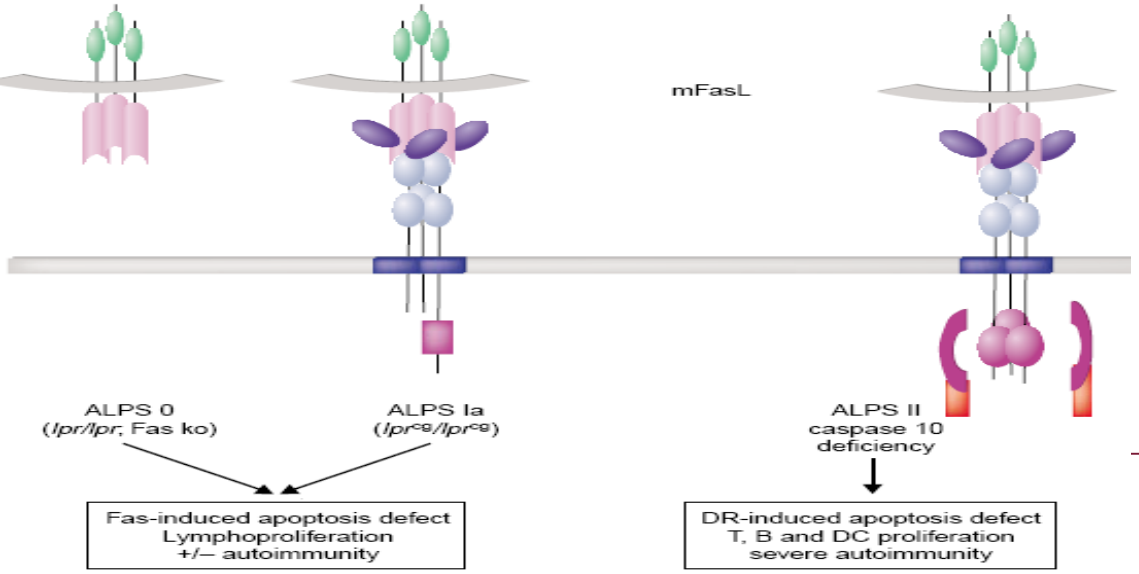
Dx: ALPS? (Autoimmune lymphoproliferative syndrom)

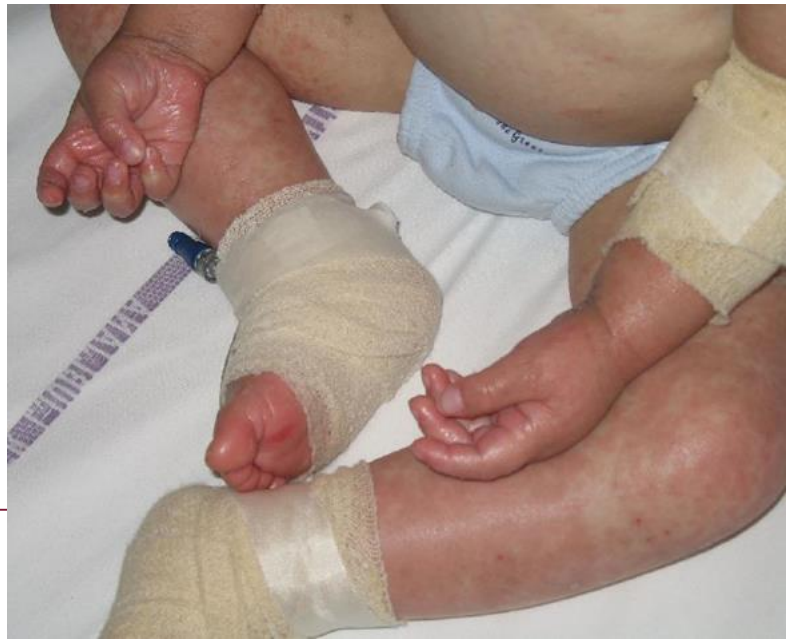
- Genetic disorder of abnormal lymphocyte survival caused by defective Fas mediated apoptosis
- Leading to chronic non-malignant lymphoproliferation, autoimmune disease, and secondary cancers

Management:

- Supportive measures
- Methylprednisolone 4mg/kg iv daily for 4 days
- Allopurinol
- Ranitidine
- Ca & vit D suppl.

good clinical and laboratory response



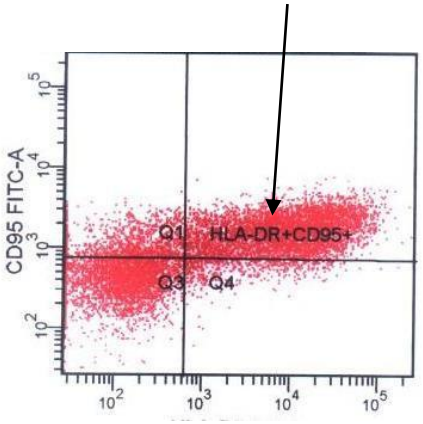


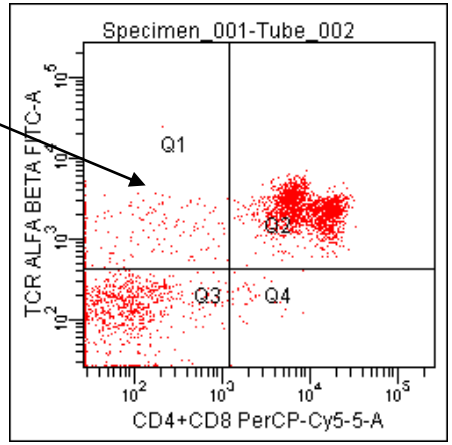
ALPS Diagnosis

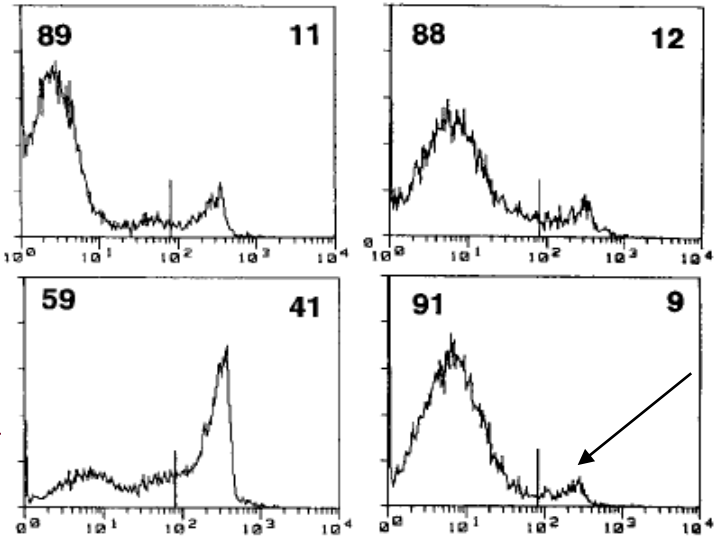
- **Elevated peripheral blood Double Negative T cells** (DNTs - CD3+/CD4-/CD8-/TCRalpha/beta+)
- Marked elevations >5% virtually pathognomonic for ALPS; mild elevations also found in other autoimmune diseases
- DNTs - thought to be cytotoxic T lymphocytes that have lost CD8 expression
- **Defective in vitro Fas mediated apoptosis**: T cells from patient and normal control supported in culture for >10 days with mitogen stimulation and IL-2 expansion and then exposed to anti-Fas IgM monoclonal antibody
- ALPS patient T cells: Do not die with anti-Fas monoclonal antibody exposure. Normal T cells from unaffected patient do.

ALPS - Elevated peripheral blood Double Negative T cells (DNTs) Required for diagnosis

- **4%** CD4-CD8- TCRα/β+ T cells
- **Normal FAS** (CD95) expression
- **Defective apoptosis** in functional test (in vitro PHA and IL-2 stimulated PBL)







Diagnostic tests:

- **appropriate Fas protein expression**
- **apoptosis test - nonrepresentative results**

Follow up

- Few small skin abscesses – treated surgically
- Pseudomonas and Staph aureus conjunctivitis
- Evident failure to thrive since 21 months
- Candida esophagitis
- Persistent refractory diarrhea (Campylobacter, Candida, Clostridium isolates)
- Persistent skin rash
- Episodes of sepsis with Staph aureus and Pseudomonas

23 months

CMV chorioretinitis and Strep. throat infection

Relapse with thrombocytopenia, AI anemia, petechiae

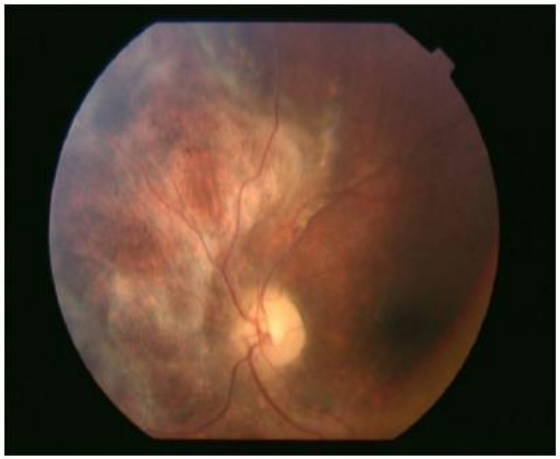
	sep.07	nov.07	dec.07	feb.08	mar.08
SR	120	20	83	92	100
CRP	9	<8	<8	50	<8
L	12,0	9,3	15,0	9,6	6,3
Hb	63	128	96	123	98
Tr	14	134	17	138	75

CD45RA	5%
CD45RO	95%
CD3+25+	25%
CD3+ FoxP3+	25%
TCR γδ+	72%
DNT (CD3+ CD4-CD8- TCRαβ+)	6%
RTE CD31+	1%

Severe CMV chorioretinitis

22 months (feb.08)

1,5 mo after introduction of MMF



CD3+	**776 ↓**	75%
CD4+	**130 ↓**	13%
CD8+	574	56%
CD19+	**56 ↓↓**	5%
CD56/16+	0.201	19%

Reduced Lymphocyte proliferation assay

Management: Gancyclovir iv. 3w - followed by valgancyclovir po.

Functional Testing of INFR, TLR and NF-κB signaling

Rezultati:

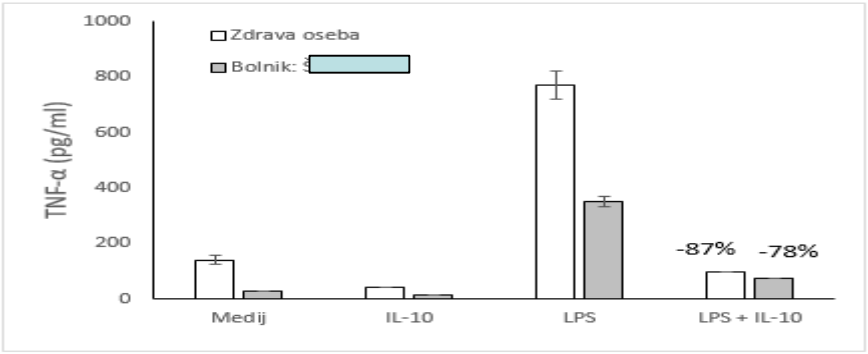
Sinteza IL-6 (pg/ml) po 48 urah:

Spodbujanje	Območje normalnih kontrol (n=15)	Bolnik/ca	Interpretacija
Medij	0-275	1	
IONO/PMA	25-1441	**14**	**ZMANJŠAN ODZIV**
IL-1β	151-5209	**86**	**ZMANJŠAN ODZIV**
Pam3CSK4 (TLR1/2)	115-7468	194	NORMALNI ODZIV
Poly(I:C) (TLR3)	5-983	7	NORMALNI ODZIV
Pam2CSK4 (TLR2/6)	324-5211	**35**	**ZMANJŠAN ODZIV**
LPS (TLR4)	622-10414	692	NORMALNI ODZIV
FLA (TLR5)	6-2861	18	NORMALNI ODZIV
CL097 (TLR7/8)	277-7294	540	NORMALNI ODZIV
CpG (TLR9)	8-764	**4**	**ZMANJŠAN ODZIV**

Rezultati:

Sinteza TNF-α (pg/ml) po 6 urah:

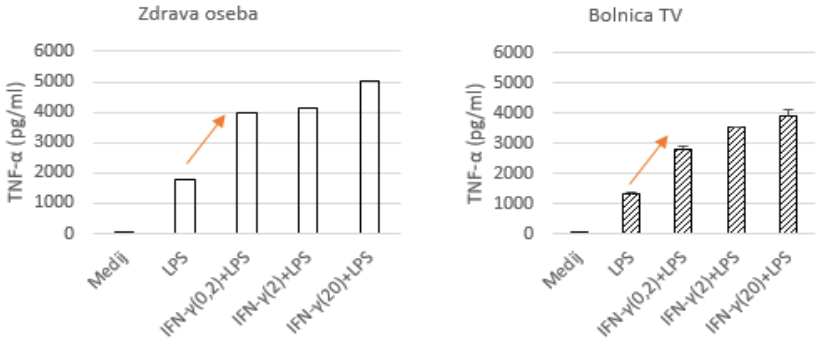
Spodbujanje	Zdrava oseba	Bolnik/ca
Medij	141	29
IL-10	41	14
LPS	771	353
LPS + IL-10	98	77



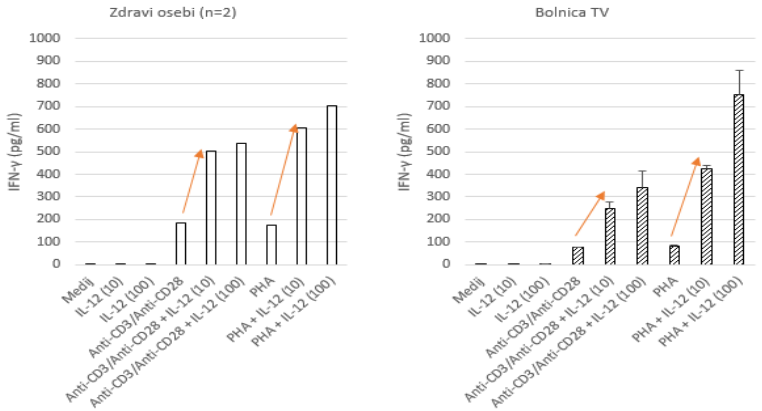
Test za določanje delovanja receptorja IFN-γR *(odvzem krvi 7.3.2017 in 4.4.2017):*

Sinteza TNF-α je bila pri bolnici pri spodbujanju z LPS in IFN-γ 2,3-krat večja, kot pri spodbujanju samo z LPS.

Rezultati NE kažejo na okvaro receptorja IFN-γR (Slika 1).



Sinteza IFN-γ po spodbujanju z anti-CD-3 in/ali z IL-12 (odvzem krvi 7.3.2017).



SCID T-B-NK+ [de Villartay et al. *J Clin Invest 2005*; 115:3291-99]

Two compound heterozygous mutations in RAG1 gene (JP de Villartay, Inserm, Paris):

- del AA368-369 [K86fs] inherited from the mother
- T1049C [C313R] inherited from the father

- **Severe complication at 3y of age:**

  Life threatening intravascular IgM mediated AI hemolytic anemia and thrombocytopenia

  - *Daily transfusions of packed RBC*
  - *SoluMedrol pulses*
  - *IVIg*
  - *Cyclosporin*
  - ***Rituximab***

Disease course

no suitable HLA matched donors

Great North Children's Hospital in Newcastle agreed to perform HSCT with best possible donor at age 3,5y

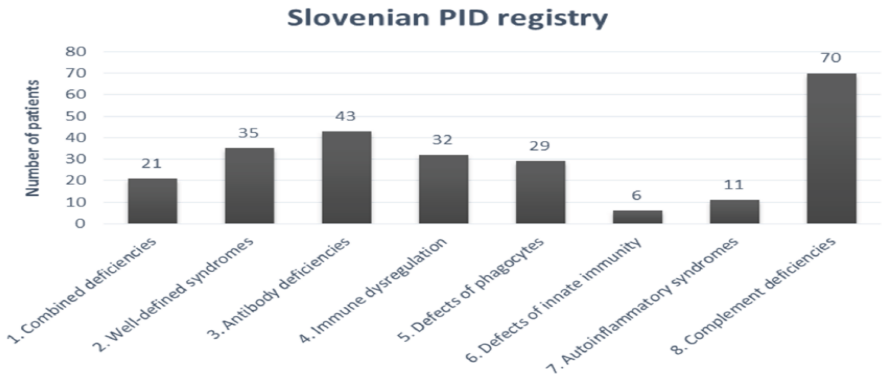
Pretransplant: pancreatitis, ARDS and refractory Stenotrophomonas sepsis, progressive liver failure, brain dysfunction and massive GI bleeding

Died at the age of 47 months.

SCID patient can present with life threatening inflammatory and autoimmune disease

Autoimmune and Inflammatory Manifestations in 247 Patients with Primary Immunodeficiency—a Report from the Slovenian National Registry

J Clin Immunol 2016 DOI 10.1007/s10875-016-0330-1



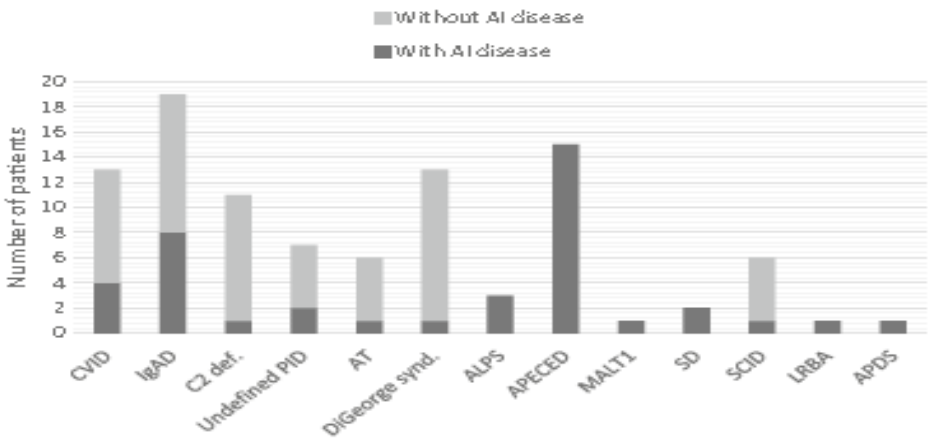


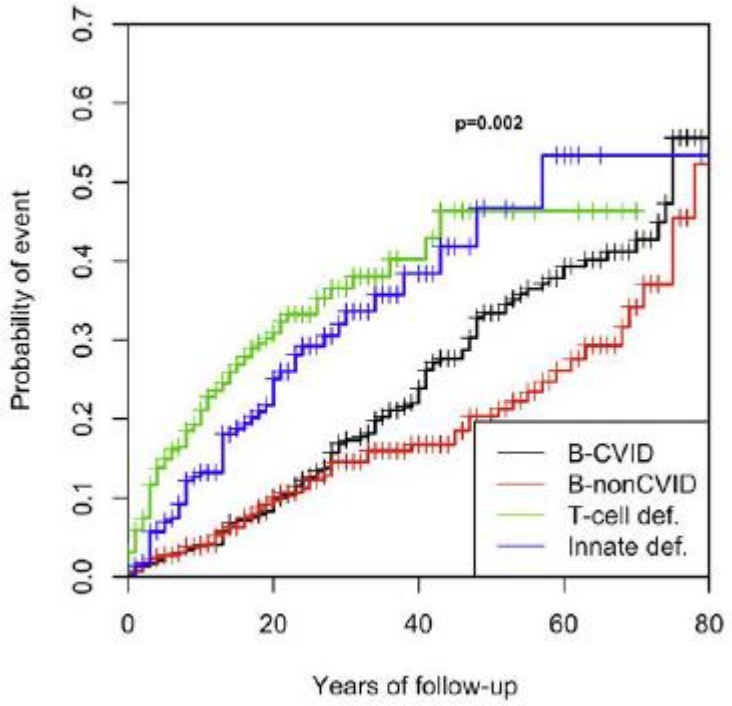
Table 1 Epidemiological data and prevalence of disease manifestations

	No. (%)
Epidemiological data	
All registered patients	247
No. of different PIDs	50
Male patients with PID	147 (59.5)
Female patients with PID	100 (40.4)
Children with PID	193 (78.1)
Adults with PID	54 (21.9)
Deceased patients	36 (14.6)
Mean age at PID onset	4.6 years
Mean age at PID diagnosis	7.6 years
Patients with genetic diagnosis	126/247 (51.0)
Disease manifestations	
All non-infectious and non-malignant	69/235 (29.4)
All patients with AI manifestation	52/235 (22.1)
AI before PID	42/52 (80.7)
AI after PID	29/52 (55.8)
All patients with lymphoprol./granul.	28/235 (11.9)
Lymphoprol./granul. before PID	22/28 (78.6)
Lymphoprol./granul. after PID	13/28 (46.4)
All patients with autoinflammation	12/247 (4.6)
All patients with allergy	10/235 (4.3)

First AI manifestation before PID diagnosis in 81% patients

Cumulative incidence of AI manifestations in French national registry

	No.	Percent
Autoimmune cytopenia	269	31.4
Gastrointestinal disorders	208	24.4
Skin	120	14.1
Rheumatologic disorders	109	12.8
Endocrine disorders	69	8.1
Lung	30	3.5
Eye	14	1.7
Kidney	11	1.3
Vasculitis and other systemic disorders	9	1.0
Neurologic disorders	8	1.0
Urologic disorders	3	0.4
Other	2	0.3
Total	852	



- Fisher et al. JACI 2017;140:1388

Defective functions in PID, leading to Autoimmunity

Mechanism	PID disease
Exacerbation of type I IFN production	Aicardi-Goutieres syndrome
Exacerbation of IL-1 production	Autoinflammatory syndromes
Defective negative selection of T/B cells	*AIRE, RAG*
Defective editing of the B cell receptor	*RAG, AID*
Defective peripheral antigen-induced death	ALPS (*FAS def.*)
Gain of function of T/B activation/efector molecules	*STAT1, STAT3, PI3K*

Mechanism	PID disease
Defective regulatory T cells	*FOXP3, CD25, STAT5b, WASP, IL-10, IL-10R, ORAI1, STIM1, BACH2,..)*
Homeostatic expansion of self reactive T cells	*RAG*
Defect in cis regulatory molecules	*CTLA4, LRBA, A20, ITCH, ROQUIN*
Defective clearance of immune complexes and apoptotic cell bodies	Complement deficiencies
Defective control of the gut microflora	CGD, *NEMO, XIAP, TTC7A,..*

- Gupta et al. Clin Rev Alerg Immun 2013

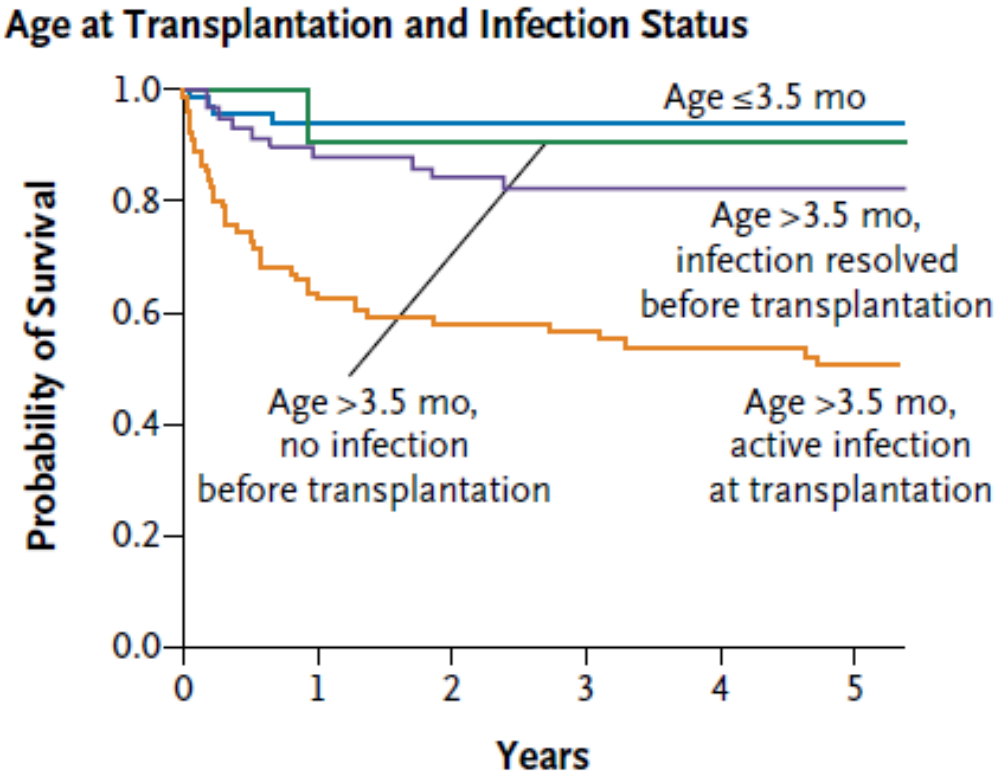
Diagnostic approach – *immunology work-up*

- Immunology tests:
  - Absolute lymphocyte count
  - Assessment of T cell subsets / T cell differentiation ‘states‘
  - Assessment of proliferation in response to mitogens and anti-CD3/CD28
  - ADA and PNP
  - Total serum immunoglobulins
  - Specific antibodies if vaccinated

Transplantation outcome for SCID

(Pai, et al, NEJM 2014; 371: 434-46)

Retrospective data from 240 infants with SCID



Case 2

- 33-year old patient with multifocal gastric carcinoma and malignant melanoma were diagnosed and surgically treated at 19 and 27 years of age, respectively.

The patient had autoimmune enteropathy, rheumatoid arthritis, megaloblastic anemia, 96% of T lymphocytes were HLA-DR activated, suggestive of a hyperactive immune state

The patient suffered from several opportunistic infections, including *Campylobacter jejuni*, *Morganella morganii*, *Proteus mirabilis*, *Yersinia enterocolitica*, *Cryptospordium parvum*, *Giardia lamblia*, *Legionella pneumophila*, pulmonary aspergillosis and aspergilloma, and chronic mucocutaneous candidiasis.

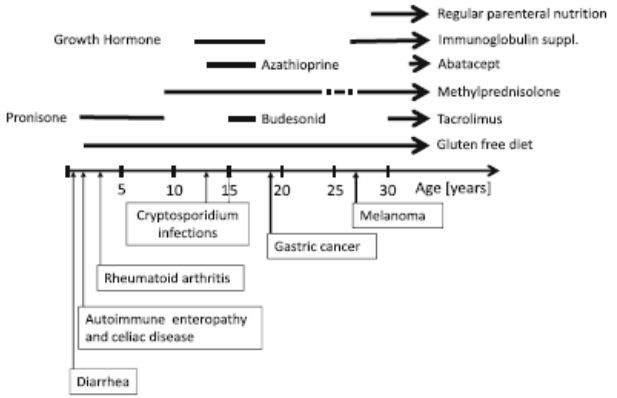


Fig. 1 Schematic representation of the course of selected treatments and age at occurrence of selected disease manifestations. Arrows indicate ongoing therapy. Broken lines indicate two periods of pulse therapy with methylprednisolone

- Whole exome sequencing (WES) was performed and phenotype driven analysis was used to direct and focus the analysis on LRBA genetic variants

A homozygous frameshift deletion in LRBA gene (p.Glu946Ter) and two common variants in TYR gene were identified.

Functional CTLA4 induction test

Reduced CTLA4 expression in a subset of regulatory T lymphocytes was identified in the patient and his unaffected mother carrying a heterozygous LRBA mutation as compared to control in a dose-dependent manner.

Bratanič et al. Orphanet Journal of Rare Diseases (2017) 12:131
DOI 10.1186/s13023-017-0682-5

Orphanet Journal of Rare Diseases

RESEARCH Open Access

Multifocal gastric adenocarcinoma in a patient with LRBA deficiency

Nina Bratanič[1], Jernej Kovač[2], Katka Pohar[3], Katarina Trebušak Podkrajšek[2,4], Alojz Ihan[3], Tadej Battelino[1,4] and Magdalena Avbelj Stefanija[1*]

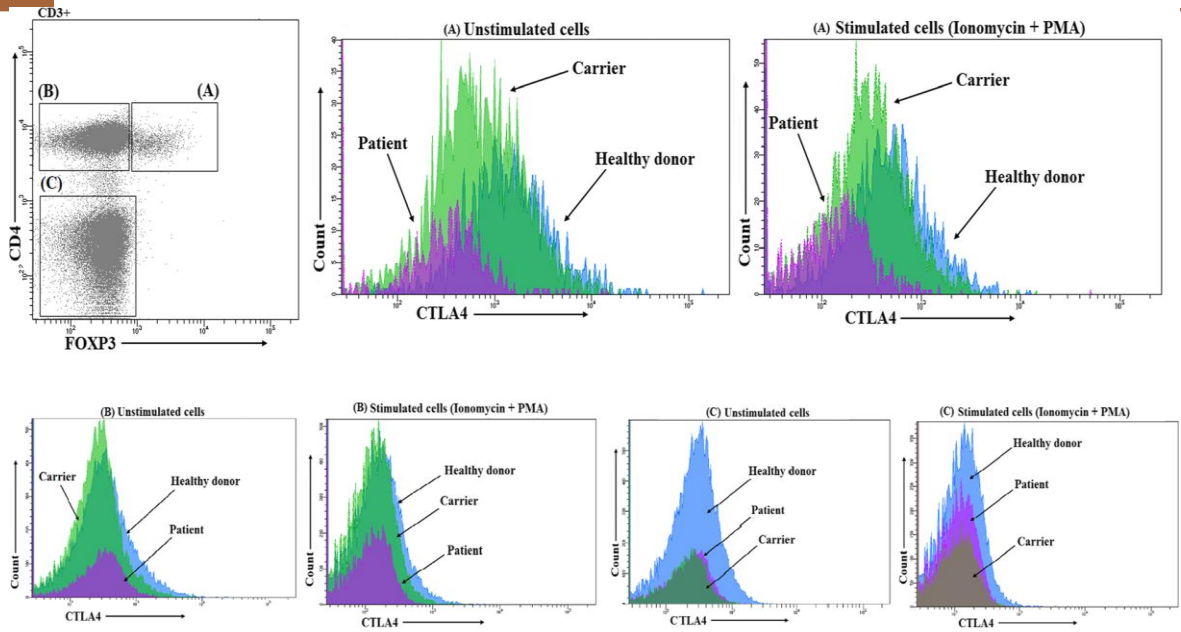
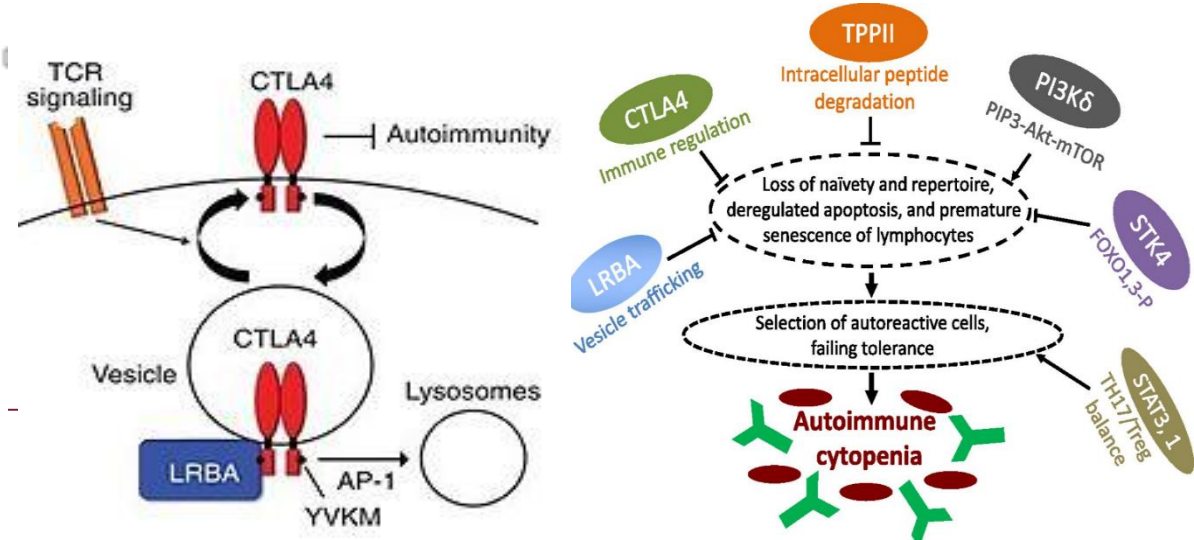


Fig. 2 Total and mobilized CTLA4 levels in CD3+ cells. Unstimulated and stimulated FOXP3+ CD4+ T cells (A), FoxP3 negative CD4 T cells (B), and CD4 negative T cells (C) from LRBA deficient patient (violet), heterozygous carrier (yellow) and healthy donor (blue). Legend: PMA – phorbol 12-myristate 13-acetate

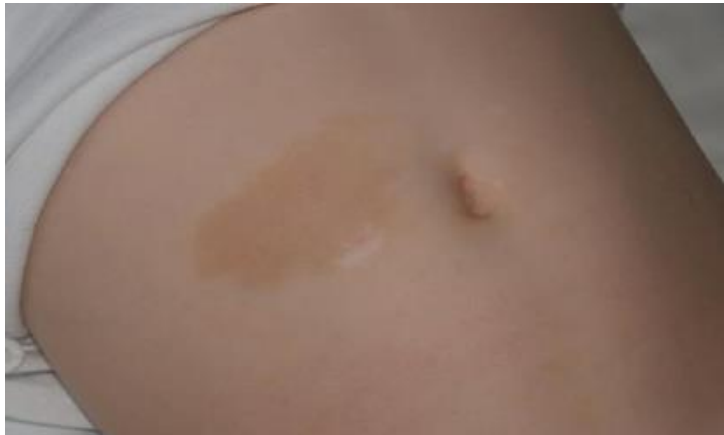


Case 3

- **Presented to our institution at 3.5y**
  - generalized lymphadenopathy and hepatosplenomegaly
  - entheropathy
  - localized skin mastocytoma
- *Infections:*
  - Since 6 mo several episodes upper respiratory tract infections (bronhitis, OMA, conjunctivitis) treated with various ATB therapy

2.5 y: IgG 2,86, IgA 0,23, IgM 1,17, IgE 4;
3 y: IgG 5,77, IgA 0,37, IgM 2,33, IgE 2;

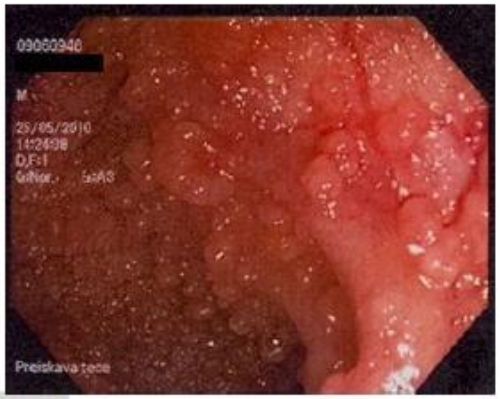
- *Lymphadenopathy:*
  - Since 3 y progressive generalised enlargement of LN, liver and spleen
- *Mastocytosis:*
  - Since 3 months local redness with vesiculaes
  - *cutaneous* mastocytoma (skin biopsy at 3y)
- *Enteropathy:*
  - Since 6 mo episodes of bloody diarrhea with colics and unusual feeling in abdomen
  - Negative serology and biopsy for coeliac disease
  - At 18 mo EDGS: folicular gastritis, chronic nonspecific duodenitis, Cryptosporidium parvum cysts

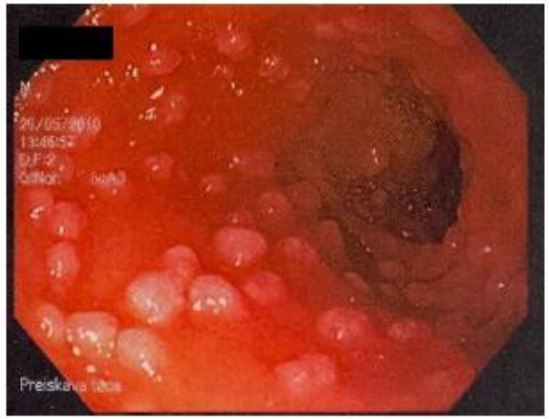


- **Imunological tests: IgG 5,22, IgA <0,28, IgM 1,74**

1,1 - 2,8	0,5 - 1,8	0,4 - 1,2	0,3 - 0,7	0,08 - 0,5	0,1 - 0,6
CD3	CD4	CD8	CD19	HLA-DR	CD16+56+
3,84	0,95	2,63	0,40	1,04	0,33

- **Unidentified etiology of lymphadenopathy**
  - CITOLOGY PUNCTION: reactive lymphadenitis with severe hyperplasia of folicular centers
  - EBV unspecific serology, PCR 554cp/ml in blood, none in LN, later <100kp/ml
  - CMV IgG pos., IgM neg.
- **GIT endoscopies:**



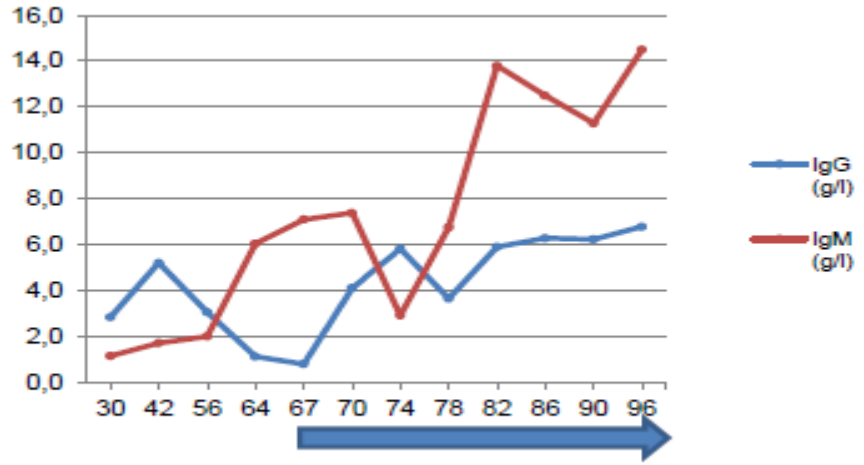


Differential Diagnosis

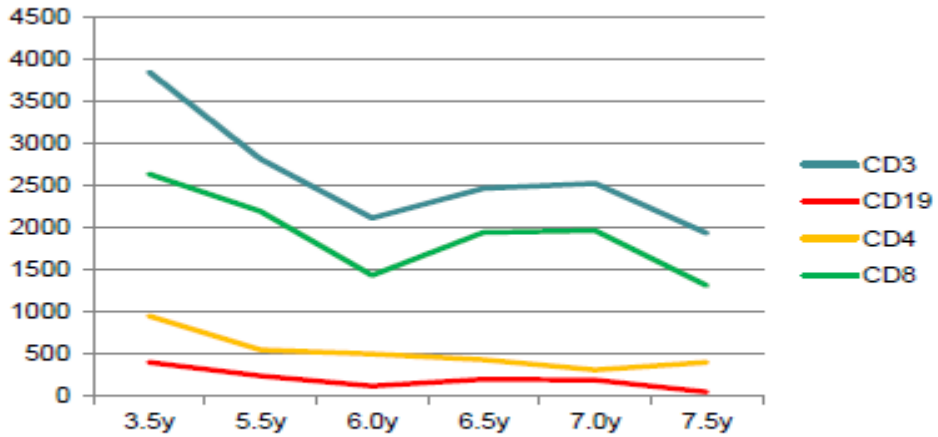
- *XLP:* lymphoproliferation, EBV infection
  - normal DNA sequence of SH2D1A and XIAP
- *ALPS:* lymphoproliferation, high DNT and IL10
  - Normal apoptosis, normal DNA sequence of FAS-L, FAS-R
- *Systemic mastocitosis:* mast cells in skin, GIT, lymphoproliferation
  - Normal Bone Marrow aspiration and biopsy
  - Normal c-KIT

Follow up:

- Exacerbations of large LN and bloody diarrhea
- In the next 4 yrs several upper respiratory tract infections with one severe pneumonia with effusion
- CMV infection with severe enlargment of LN
- Chronic dacryocystitis
- Severely enlarged liver with signs of liver fibrosis
- EBV colitis
- Progressive leucopenia, AI anemia



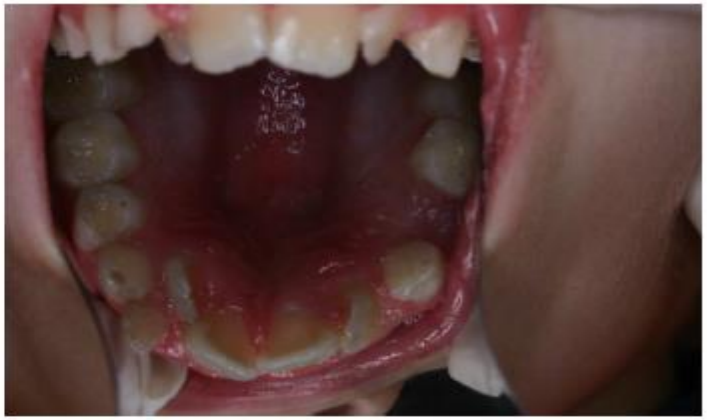
IVIg suplementation



Low RTE, CD45RA

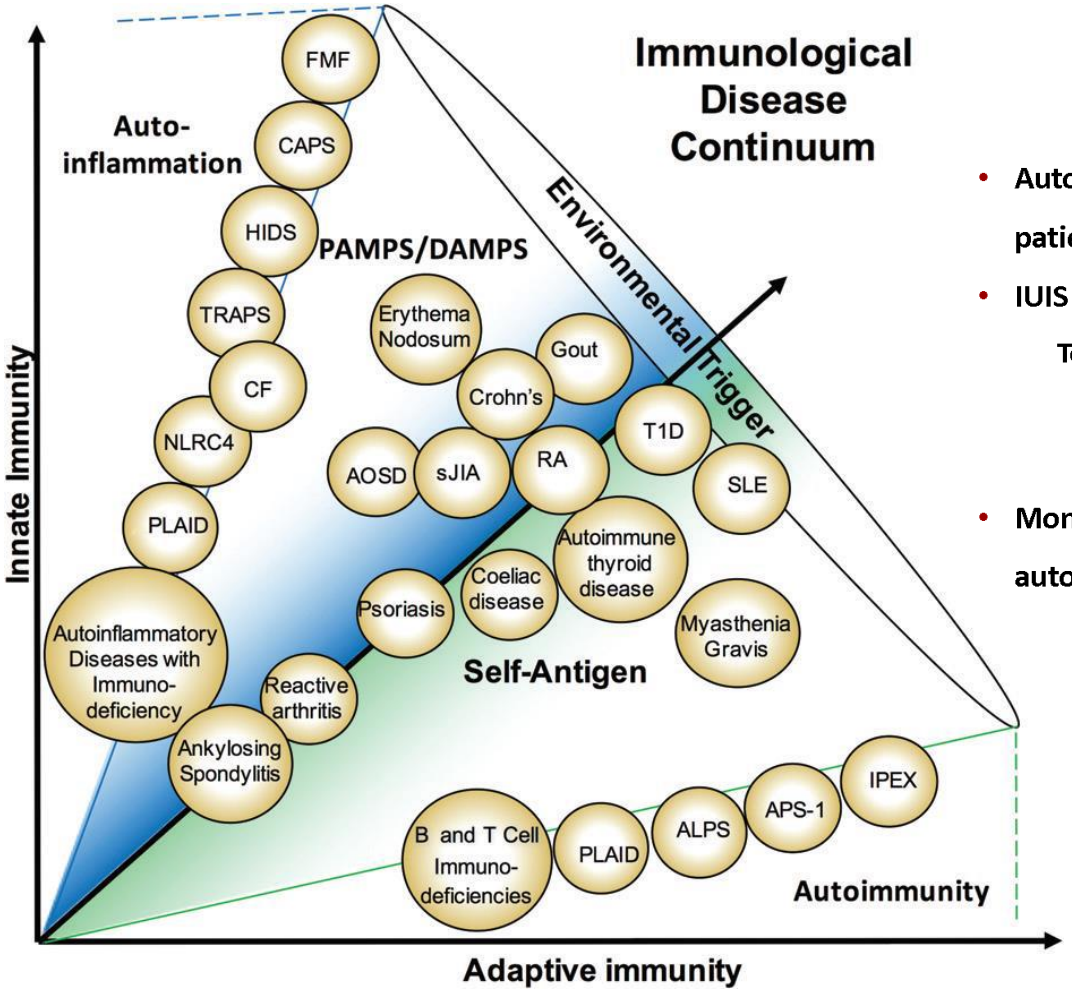
- **Hyper IgM syndrome:**
  - Low CD40L (CD154) expression
  - normal DNA sequence of CD40L, AICDA, UNG
  - normal RAG1 sequence
  - RAG2 and NEMO negative

- **Therapy:**
  - TMP/SMX prophylaxis
  - IVIg 1g/kg every 3 wks
  - Th for mast cell infiltration



  - Underwent successful BMT in Newcastle upon Tyne, UK

Immunological disease continuum



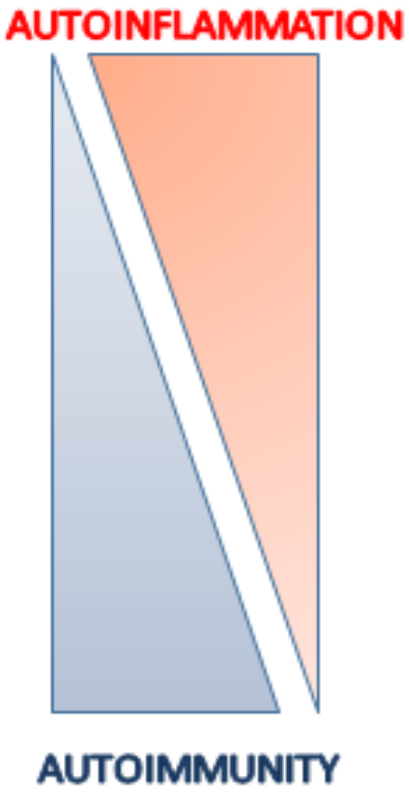
- Autoimmune manifestations frequent and often multiple in patients with PID
- IUIS classification for PID (Bousfiha, et al. J Clin Immunol 2015)

  Total 320 diseases
  - 146 (45%) monogenic PID causing autoimmunity and/or autoinflammation
- Monogenic PID provide unique insight into pathogenesis of autoimmunity

Peckham J Pathol 2017;241:123

The autoinflammatory disorders (AID) are a new and expanding classification of inflammatory diseases

- Recurrent episodes of systemic inflammation
- NO pathogens, NO autoantibodies, NO autoreactive T cells



TRAPS

FCAS

HIDS

CROHN'S DISEASE

GOUT

BEHCET'S SYNDROME

UVEITIS

RHEUMATOID ARTHRITIS

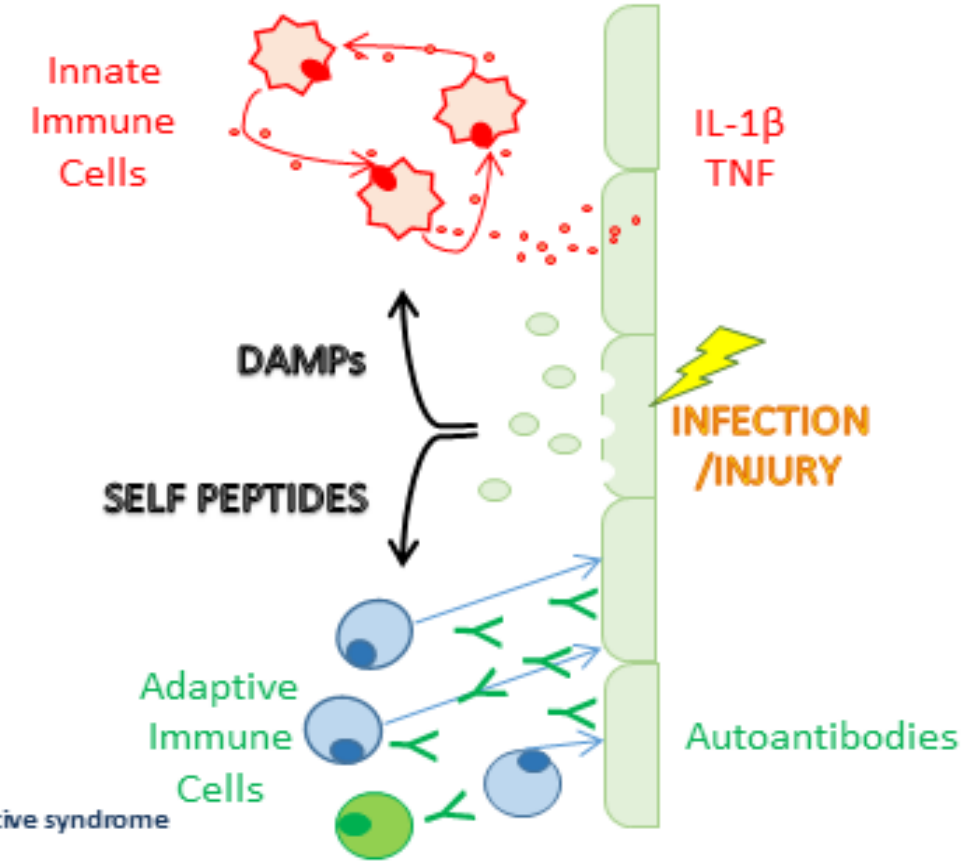
TYPE-1 DIABETES

SJORGREN SYNDROME

SYSTEMIC LUPUS ERYTHEMATOSUS

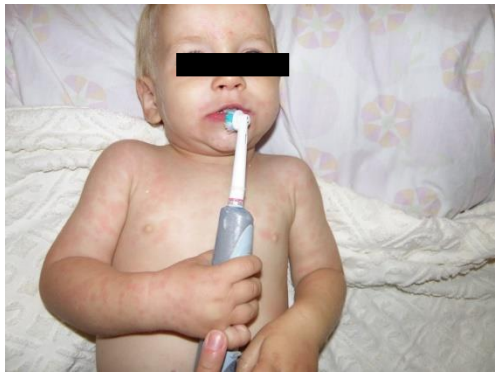
Autoimmune lymphoproliferative syndrome

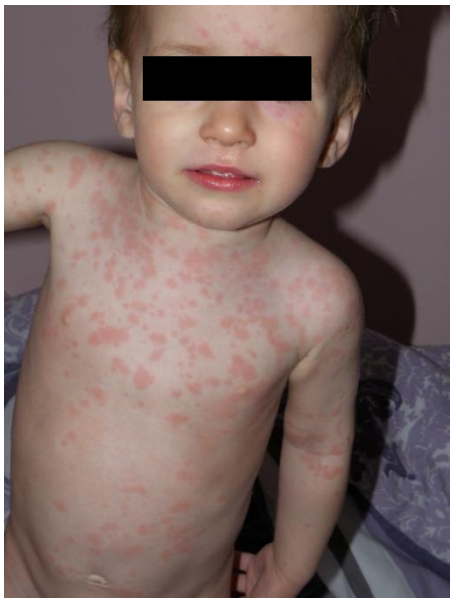
IPEX



Case 4

- Male, caucasian, gestation age 37 weeks, neonatal sepsis suspected, not proven, antibacterial treatment
- **Urticarial rash- appeared soon after birth**
- He had the first episode of **fever with elevated CRP (50)** and no signs of an infection at the age of six months, **fever lasted for four days (no infection was proven)**
- First episode of **limping at the age of 16 months, arthritis of a hip disappeared in 24 hours**





Urticarial rash-

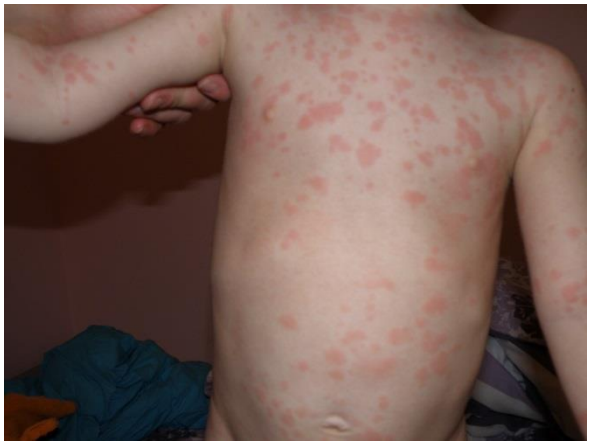
appeared soon after birth, was never itchy, was more intense during fever, but didn't disappeared between fever attacks

Slide courtesy of N Toplak

Case 4

MAIN PROBLEMS

- **periodic fevers** from 6 month of age, **short attacks** 1-2 days, sometimes up to 5 days, every 7-30 days
- **elevated inflammatory parameters** (CRP up to 200), between attacks inflammatory parameters also elevated- CRP from birth never below 40
- **Arthritis** from 16 month of age - last 1-2 days, sometimes red joint as in FMF, fluid in hip joints, knees, ankle- not all of them in every attack
- **Rash-** looks like urticarial, sometimes more macular; rarely disappears completely; in milder form between attacks present all the time; mastocyts found in skin biopsy;



systemic mastocitosis excluded

by bone marrow biopsy

Diagnostic approach

- Exclusion of infections
- Exclusion of PID

- Exclusion of autoimmune diseases autoantibodies negative: ANA negative, ANCA negative, antiC1 Q- negative
- Exclusion of allergy milk, egg,triptase normal on several occasions
- Exclusion of malignant disease bone marrow biopsy

- Metabolic disease?

Organic acid in urine

2011 - negative 4 times in febrile attacks, **no mevalonic acid**

- Complemet levels- classic, alternative activation normal
- IgG, IgM, IgA normal

IgD 324E/ml (normal up to 90)

- Flow cytometry- normal

- Exclusion of chronic granulomatosis
- Titre of anti-diphteria antibodies 0,292 IU/ml (protected)
- Titre of anti-tetanus antibodies 0,350 IU/ml (protected)

s-SAA ELISA 480 mg/L (N<6,4)

Slide courtesy of N Toplak

frontiers in IMMUNOLOGY

CLASSIFICATION ARTICLE
published: 22 April 2014
doi: 10.3389/fimmu.2014.00162

Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency

Waleed Al-Herz[1,2], Aziz Bousfiha[3], Jean-Laurent Casanova[4,5], Talal Chatila[6], Mary Ellen Conley[4], Charlotte Cunningham-Rundles[7], Amos Etzioni[8], Jose Luis Franco[9], H. Bobby Gaspar[10], Steven M. Holland[11], Christoph Klein[12], Shigeaki Nonoyama[13], Hans D. Ochs[14], Erik Oksenhendler[15,16], Capucine Picard[5,17], Jennifer M. Puck[18], Kate Sullivan[19] and Mimi L. K. Tang[20,21,22]*

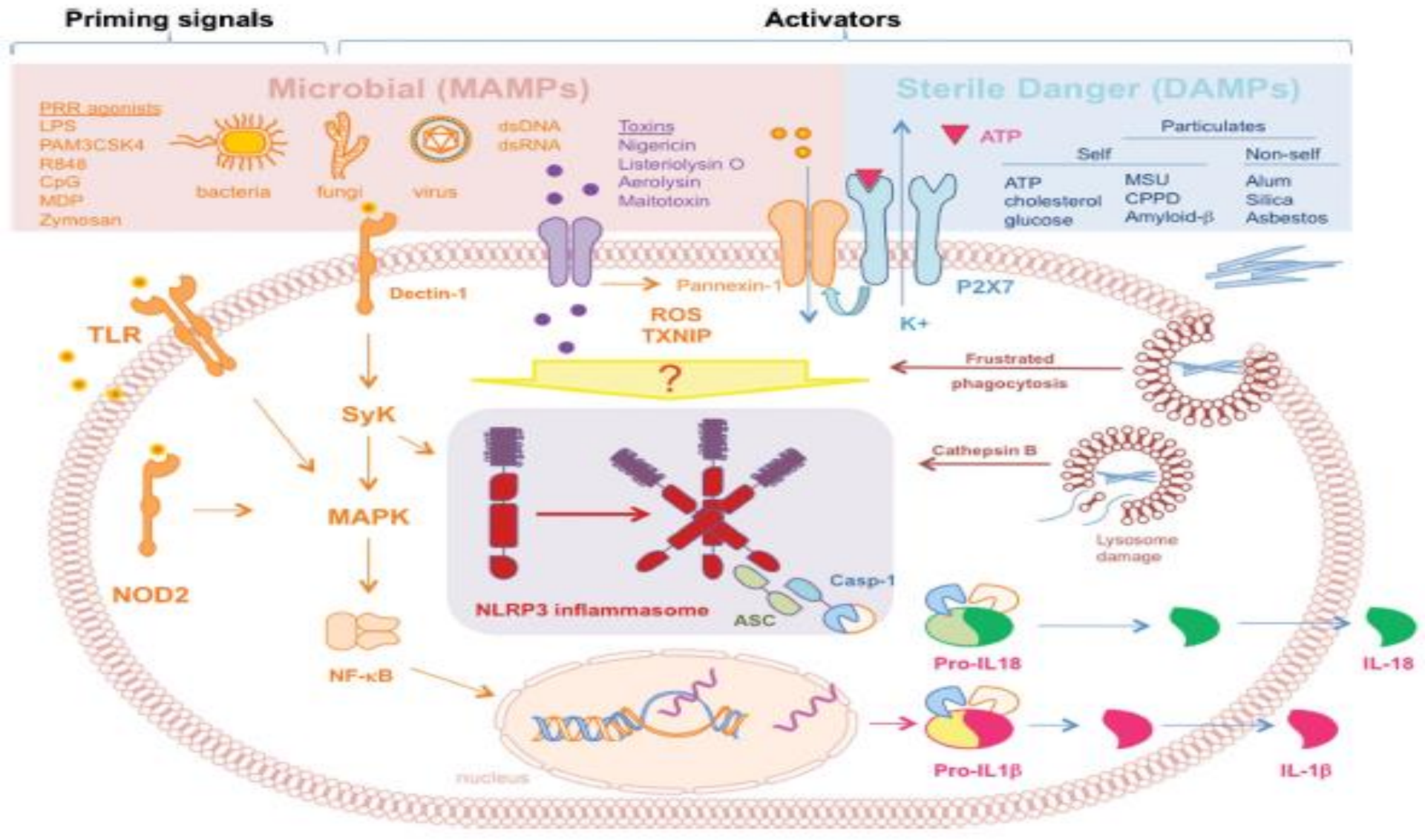
CATEGORIES- major groups of PID
1. Combined ID- SCID
2. Combines ID with associated syndromic features
3. Predominantly ab deficiencies
4. Diseases of immune dysregulation / type 1 interpheronopathies
5. Congenital defects of phagocyte number, function or both
6. Defects in innate immunity
7. Autoinflammatory disoreders
8. Complement deficiencies
9. Phenocopies of PID (acquired defects)

The Molecular Pathophysiology of Autoinflammatory Disease

	Provisional molecular/ functional classification of AID	Disease
1	**IL-1β activation disorders (inflammasomopathies)**	Intrinsic: **FCAS, MWS, CINCA / NOMID** Extrinsic: **FMF**, PAPA, CRMO / SAPHO, Majeed sy, **HIDS, DIRA**, recurrent hydatiform mole Complex /acquired: gout, DM II, Schnitzler sy., fibrosing disorders
2	**NF-B activation disoreders**	Crohn's disease, Blau sy, **FCAS2- Guadalupe periodic fever- NALP 12**
3	**Protein folding disorders of the innate immune system**	**TRAPS**, spodyloarthropathies
4	**Complement disorders**	aHUS, AMD- age related macular deg.
5	**Cytokine signaling disorders**	Cherubism
6	**Macrophage activation**	fHLH, Chediak-Higashi sy.,Griscelli sy., X-linked lymphoproliferative sy., Hermansky-Pudlak sy., secHLH, atherosclerosis

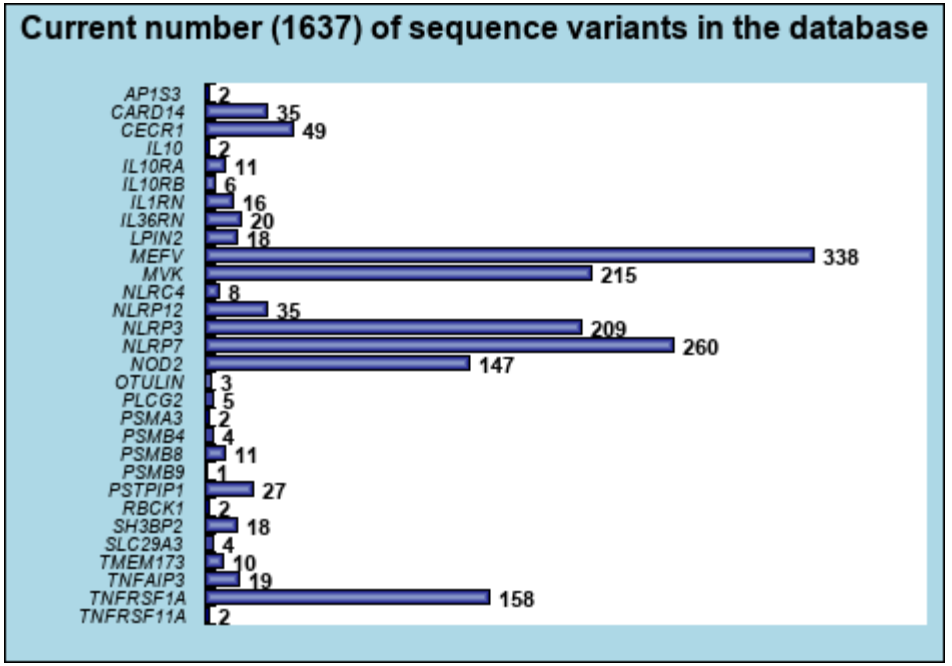
Set L. Masters et al. Annu Rev Immunol. 2009

MAMPs & PAMPs signaling



Hereditary periodic fever syndromes

- **Familiar Mediterranean fever - FMF**
- Hyperimunoglobulinemia D - HIDS / mevalonate kinase deficiency- **MKD**
- TNFαR periodic fever syndrome - **TRAPS**
- Criopyrin associated periodic fever syndormes- **CAPS**
  - *Familial Cold Autoinflammatory Syndrome-FCAS/ Familial Cold Urticaria Syndrome- FCUS*
  - *Muckle-Wells syndrome-MWS*
  - *Neonatal-Onset Multisystem Inflammatory Disease-NOMID/Chronic Infantile Neurologic, Cutaneus and Articular Syndrome-CINCA*



http://fmf.igh.cnrs.fr/ISSAID/infevers/

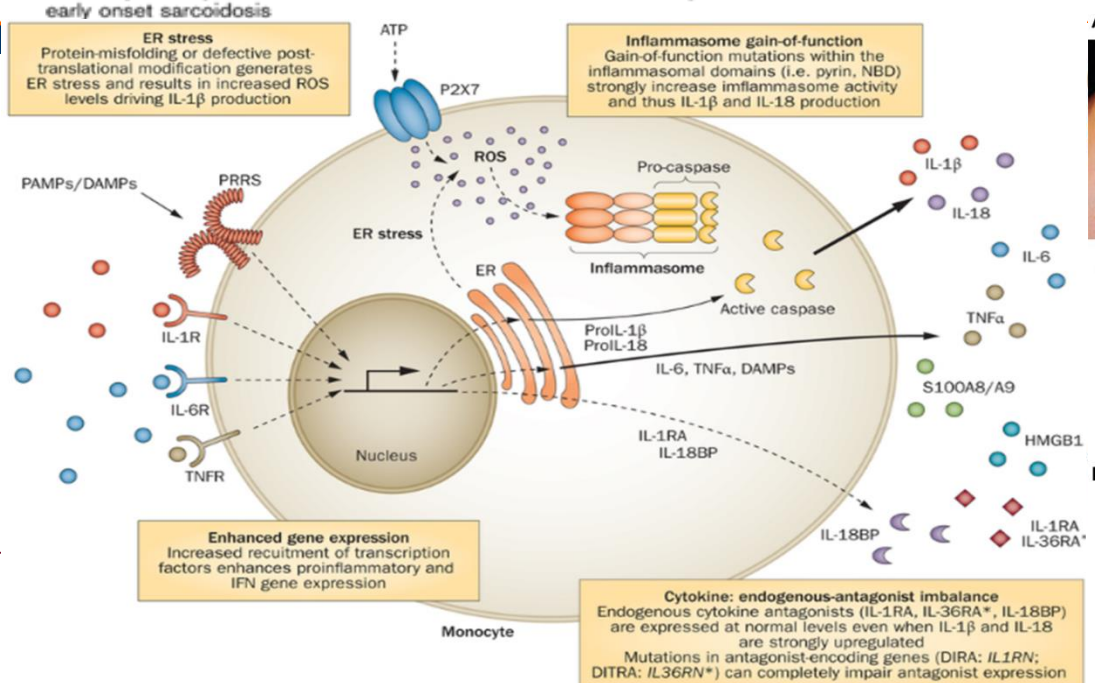
International Society for Systemic AutoInflammatory Diseases

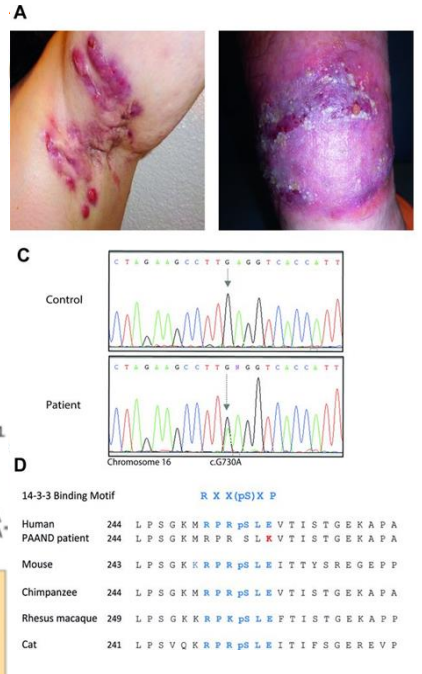
Periodic Fever Syndromes

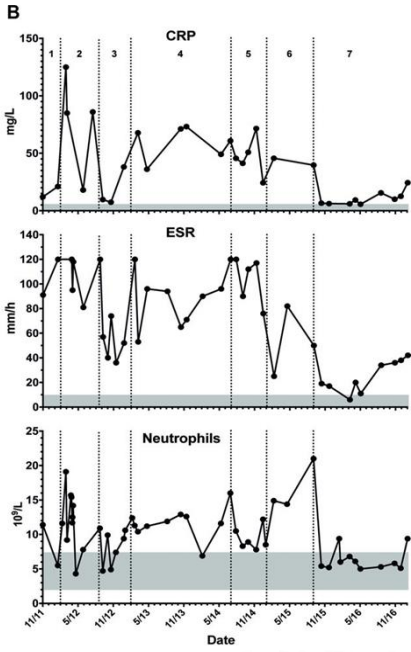
Sporadic conditions- PFAPA

Hereditary conditions: FMF, MKD/HIDS, TRAPS, CAPS

	Inheritance	Underlying gene	Duration of attacks	Clinical features (major)	Treatment
Familial Mediterranean fever	Autosomal recessive	*MEFV*- encoding pyrin	1–3 days	Fever with peritonitis, erysipeloid erythema, and monoarthritis	Daily colchicine, anti-TNF or IL-1 receptor antagonist (investigational)
Hyper IgD syndrome	Autosomal recessive	*MVK* –encoding mevalonate kinase	3–7 days	Fever, maculopapular rash, diarrhea, abdominal pain	Investigational use of montelukast, anti-TNF or IL-1 therapy
TNF receptor-associated periodic syndrome	Autosomal dominant	*TNFRSF1A*- encoding p55 TNF receptor	Days to weeks	Fever, peritonitis, large joint arthritis, periorbital edema	Corticosteroid, anti-TNF therapy
Cryopyrin-associated periodic syndromes					
Familial cold autoinflammatory syndrome	Autosomal dominant	*CIAS1/NLRP3* encoding cryopyrin	Up to 1 day	Cold-induced urticarial rash, arthralgia and conjunctivitis	IL-1 receptor antagonist
Muckle-Wells syndrome	Autosomal dominant	*CIAS1/NLRP3* encoding cryopyrin	Several days or continuous	Urticarial rash, deafness, conjunctivitis, and arthritis	IL-1 receptor antagonist
Neonatal-onset multisystem inflammatory disease	Majority are *de novo*	*CIAS1/NLRP3* encoding cryopyrin	Continuous	Urticarial rash, epiphyseal overgrowth, meningitis, mental retardation, deafness	IL-1 receptor antagonist
Pyogenic arthritis with pyoderma gangrenosum and acne	Autosomal dominant	*PSTPIP1*	Episodic lasting weeks to months	Severe cystic acne, pyoderma gangrenosum, arthritis	Corticosteroids, IL-1 receptor antagonist or anti-TNF therapy
Pediatric granulomatous arthritis including Blau syndrome and early onset sarcoidosis	Autosomal dominant/*de novo*	*NOD2/CARD15* encoding NOD2	Chronic inflammation	Granulomatous inflammation of joints, eyes and skin, rarely affects lung	Corticosteroids, anti-TNF therapy (investigational)







The syndrome of Hemophagocytic Lymphohistiocytosis in primary immunodeficiencies

- 63 patients with primary immunodeficiencies other than cytotoxicity defects or X-linked lymphoproliferative disorders, presenting with conditions fulfilling current criteria for hemophagocytic lymphohistiocytosis.

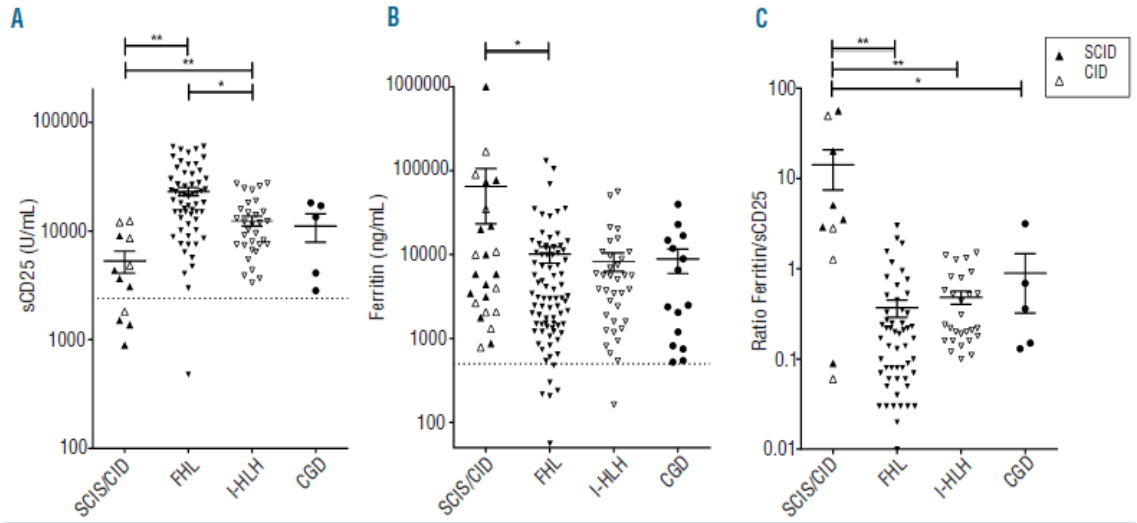


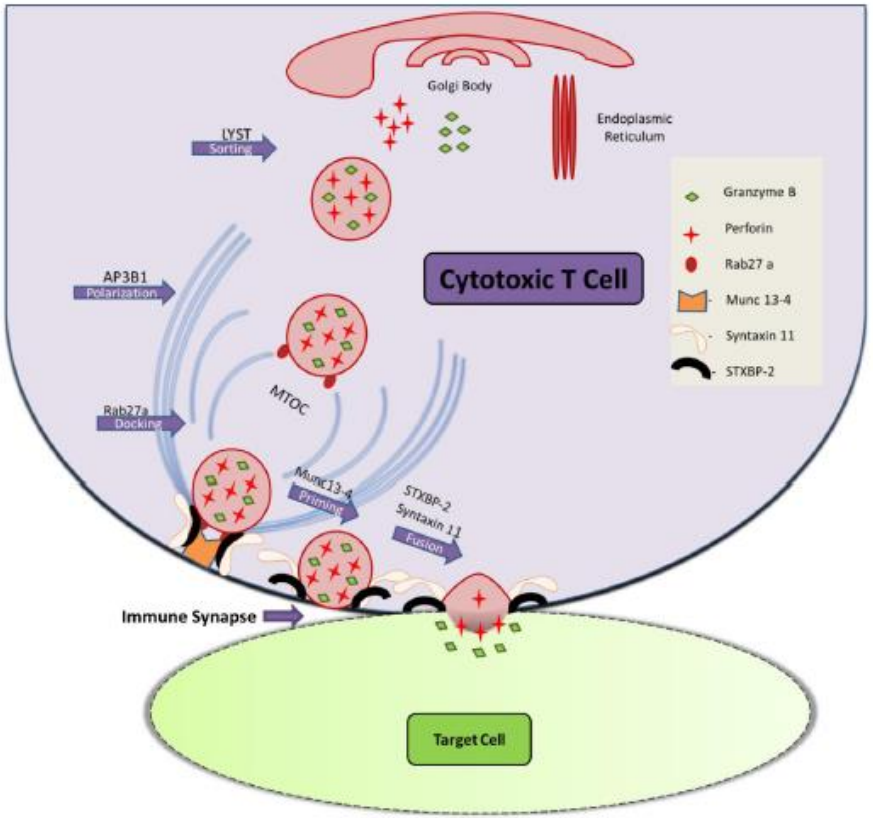
Figure 1. (A) Serum sCD25 levels in patients with SCID/CID, FHL, I-HLH (infection triggered "secondary" HLH), and CGD. (B) Serum ferritin levels in SCID/CID, FHL, I-HLH, and CGD patients. (C) Ratio of ferritin/sCD25 in SCID/CID, FHL, I-HLH, and CGD patients. *P<0.05. **P<0.01. The dotted line indicates the cut-off value according to the HLH diagnostic criteria. The bars indicate means ± standard deviation.

- Twelve patients had severe combined immunodeficiency with <100/µL T cells, 18 had partial T-cell deficiencies; episodes of hemophagocytic lymphohistiocytosis were mostly associated with viral infections.
- Twenty-two patients had chronic granulomatous disease with hemophagocytic episodes mainly associated with bacterial infections.
- Compared to patients with cytotoxicity defects, patients with T-cell deficiencies had lower levels of soluble CD25 and higher ferritin concentrations.

Bode S, Haematologica | 2015; 100(7)

Genetic defects involved in granule-mediated cytotoxicity leading to Hemophagocytic Lymphohistiocytosis (HLH)

- CTL activation results in microtubule organizing Centre (MTOC) polarization and transport of cytotoxic granules.
- LYST and AP3B1are involved in sorting and transport of cytotoxic granules.
- The granules are then docked to the site of immune synapse by Rab27a.
- Granule priming is mediated by Munc13-4, and membrane fusion by STX11 and STXBP2.
- Granule fusion results in perforin mediated pore formation and release of lysosomal enzymes leading to target cell death.
- Genetic defects in highlighted proteins involved in granule-mediated cytotoxicity leads to HLH.



The Journal of Pediatrics 2013;163:1253–1259

Diagnostic criteria for HLH

- **Diagnosis of HLH is based on the presence of 5 or more of the following**: Fever; Splenomegaly; Cytopenias (affecting 2 of 3 lineages in the peripheral blood); Hemoglobin <90 g/L (<100 g/L in infants aged <4 weeks); Platelets <100 109/L; Neutrophils <1.0 109/L; Hypertriglyceridemia and/or hypofibrinogenemia; Fasting triglycerides >3.0 mmol/L; Fibrinogen <1.5 g/L; Hemophagocytosis in bone marrow, spleen, or lymph nodes; Low or absent NK-cell activity; Ferritin >500 mg/L; sIL-2R >2400 U/mL

HLH type	Defective gene	Function	Notable clinical findings	Rapid diagnosis by flow cytometry
FHLH-2	*PRF1*	Pore formation		Decreased/absent perforin expression
FHLH-3	*UNC13D*	Vesicle priming	Increased incidence of CNS HLH	Decreased CD107a expression
FHLH-4	*STX11*	Vesicle fusion	Mild, recurrent HLH, and colitis	Decreased CD107a expression
FHLH-5	*STXBP2*	Vesicle fusion	Colitis and hypogammaglobulinemia	Decreased CD107a expression
Syndromes				
Griscelli syndrome type II	*RAB27A*	Vesicle docking	Partial albinism and silvery-gray hair	Decreased CD107a expression, abnormal hair shaft examination*
Chediak-Higashi syndrome	*LYST*	Vesicle trafficking	Partial albinism, bleeding tendency, and recurrent pyogenic infection	Decreased CD107a expression, abnormal neutrophil granules†
Hermansky-Pudlak syndrome type II	*AP3B1*	Vesicle trafficking	Partial albinism, bleeding tendency, and immunodeficiency	Decreased CD107a expression
EBV-driven				
XLP-1	*SH2D1A*	Signaling in T, NK, and NK T-cells	Hypogammaglobulinemia and lymphoma	Decreased/absent SAP expression
XLP-2/XIAP‡	*BIRC4*	Signaling pathways involving NF-κB	Mild, recurrent HLH and colitis	Decreased/absent XIAP expression
IL-2–inducible T-cell kinase deficiency	*ITK*	Signaling in T-cell	AR, Hodgkin lymphoma	NA (gene sequencing required)
CD27 deficiency	*CD27*	Lymphocyte costimulatory molecule	AR, combined immunodeficiency	Absent CD27 expression on B cells
XMEN	*MAGT1*	T-cell activation via T-cell receptor	Combined immunodeficiency, chronic viral infections, and lymphoma	Decreased CD4 cells and defects in T-cell receptor signaling

Case

- 21-year-old Caucasian male
- Presented with a weeklong history of fever, sore throat, myalgia, arthralgia and non-erythematous, papular rash to the chest and bilateral upper and lower extremities.
- Rapid strep test returned negative.
- His symptoms worsened, and the rash progressed.
- Throat cultures returned positive for Group A strep, and he was started on amoxicillin
- **CT of abdomen and pelvis**: ***Splenomegaly.***
- All blood cultures were no growth, and respiratory viral panel was negative.
- EBV IgG positive, but IgM and PCR were negative.
- CMV negative

Hospital Course

Upon admission to internal medicine for GAS and possible acute rheumatic fever, he continued to spike fevers, desaturated to 80%, and became hypotensive.

Broad-spectrum antibiotics were started, and she was intubated.

He received IVIG for toxic shock syndrome with mild to modest improvement in the appearance of maculopapular rash.

Developed multi-organ failure (AKI, hepatic failure, DIC, congestive heart failure with elevated troponin and evidence of pericarditis/carditis)

Required renal replacement therapy, packed RBC transfusion, FFP, spike temperatures and leukcoytosis worsen despite of broad spectrum Antibiotics

Rheumatology consulted for Autoimmune disease vs macrophage activation syndrome

Pediatric hematology oncology consulted for possible hemophagocytic lymphohistiocytosis (HLH)

- WBC 21,000
- BUN/Cr 18/2.1
- H/H 11/33
- Troponin 0.263
- lactic acid 1.1
- BNP 968
- LDH 1366
- ESR 45
- CRP 384.

Hospital Course

Subsquent blood work reveals the following

Ferritin 26000 ng/mL

TG 455 mg/dL

Absent NK cell activity

soluble CD25 (soluble IL-2 receptor alpha) 13630 pg/mL

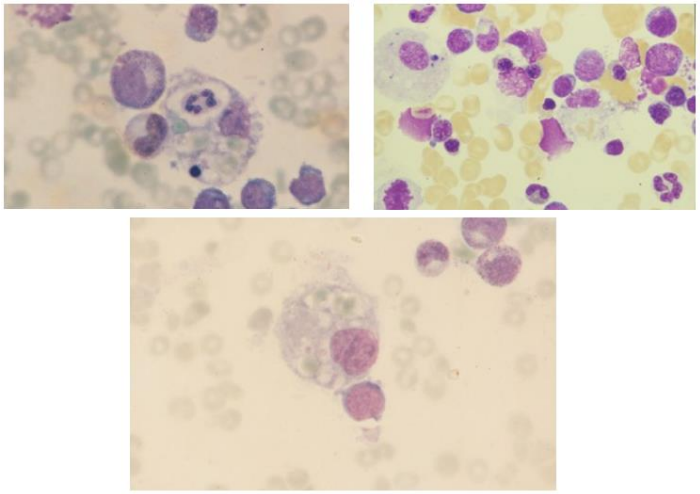
Some Peripheral smears (hematophagocytosis)

BM biopsy negative for malignancy and no evidence of HLH

BM Chromosomal analysis: **No clonal abnormality.**

HLH diagnosis made based on HLH guideline criteria

Patient started on daily high dose Dexamethasone
Significant improvement noticed, fever subsided, leukocytosis resolved



Guidelines set by **HLH**:

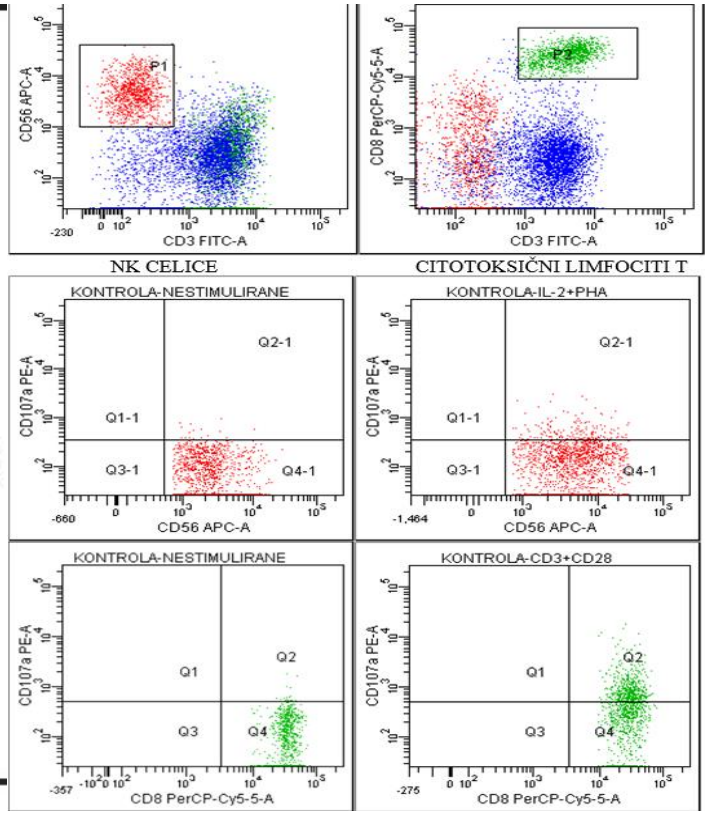
The diagnosis of HLH can be established if one of either one or two is fulfilled

- A molecular diagnosis consistent with HLH
- *or*
- Diagnostic criteria for HLH fulfilled (five out eight criteria)

At least 5 out of the 8 below to be fulfilled

- Fever
- Splenomegaly
- Cytopenias (affecting ≥2 of 3 lineages in the peripheral blood with hemoglobin <10 g/dL, platelets <100×10⁹/L and neutrophils < 1.0×10⁹/L)
- Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides >265 mg/dL, fibrinogen ≤1.5 g/dL)
- Hemophagocytosis in bone marrow or spleen or lymph nodes[$]
- Low or absent NK-cell activity (according to local laboratory)[@]
- Ferritin ≥500/µg/dL
- Soluble CD25 (Soluble Interleukin-2 receptor) ≥2400 U/mL*

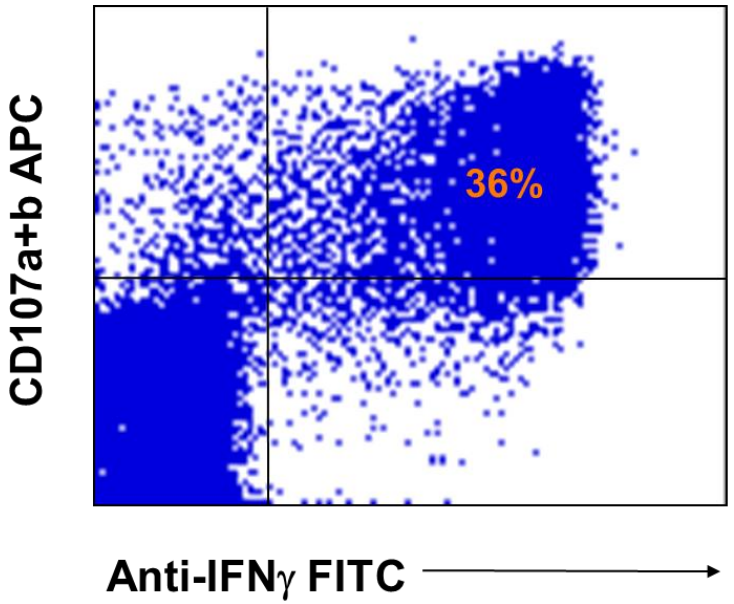
[$] Hemophaogcytosis may be absent at initial evaluation; repeated marrow and/or tissue aspirates and biopsies may be needed,*Is the most sensitive biochemical test and levels co-relate with response and prognosis; levels are age-dependent and local data needs to be ascertained, [@]Sustained low NK cell activity suggests perforin and/ or granzyme pathyway abnormality and screening for CD107a (LAMP-1 cell surface expression by cytometry) expression is recommended; NK-cell activity may be transiently depressed in IAHS. Persistent NK-cell activity depression even in the face of clinical resolution and absent known mutations should trigger referral for bone marrow transplantation



	PREISKOVANEC (%)	KONTROLA (%)
NESTIMULIRANE CELICE		
Delež celic CD107a med celicami NK (CD56)	1,3	1,9
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	8,0	7,5
STIMULACIJA CELIC Z IL-2 IN PHA		
Delež celic CD107a med celicami NK (CD56)	4,4	20,4
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	24,4	25,4
STIMULACIJA CELIC S CD3 IN CD28:		
Delež celic CD107a med celicami NK (CD56)	2,6	11,7
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	33,4	27,5

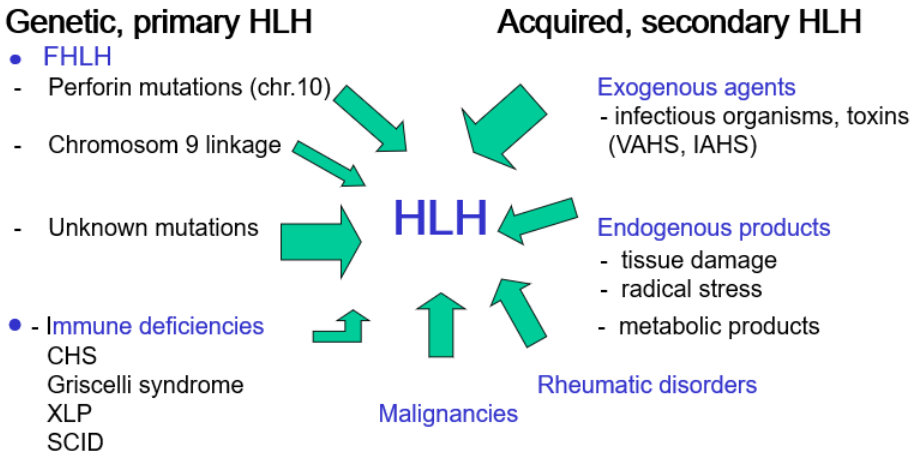
CD107 Assays

- CD107a and CD107b are proteins found in cytotoxic granules of CTL and other cells
- Upon degranulation, CD107a and CD107b are transiently transported to the cell surface
- Using labeled antibodies to CD107a and CD107b during short-term stimulation, the exocytosis of CD107 is captured on degranulating cells.
- Compare BAT – CD63



Differential Diagnosis

- Infection/sepsis
- Malignancies
  - (leukemia, lymphoma, other solid tumors)
- Drug reaction with eosinophilia and systemic symptoms (DRESS)
- Autoimmune lymphoproliferative syndrome (ALPS)
- Adult Still's Disease
- Macrophage activating syndrome



Secondary HLH to Group A strep infection

Case

- 51-yr male, healthy;
- Presented with a fever, myalgia, arthralgia His symptoms worsened
- 25.10.2017 doksiciklin;
- 29.10. – 20.11. hospitalisation (Clinic for Infectious dis); fever, CRP 90;

doksiciklin 10 days, no improvement

- pancitopenia, elevated LDH, ferritin, CRP, ESR
- CT of abdomen and pelvis: Splenomegaly.
- All blood cultures were no growth
- US: hepatomegaly, enlarged lymph nodes,
- BM biopsy negative for malignancy
- BM Chromosomal analysis: No clonal abnormality
- HLH diagnosis made based on HLH guideline criteria

- Fever > 38,5°C
- Splenomegaly
- Citopenia
  - hemoglobin 82 g/L(<90 g/L);
  - thromb < 100 x 10^9/L;
  - neutro < 1,0 x 10^9/L;
- Hypertrigliceridemia 3,1 mmol/L
- Absent NK cell activity
- soluble CD25 (soluble IL-2 receptor alpha) 7530 U/mL (≥ 2400 U/mL)
- Feritin >16000 µg/L (≥ 500 µg/L)

	PREISKOVANEC (%)	KONTROLA (%)
NESTIMULIRANE CELICE		
Delež celic CD107a med celicami NK (CD56)	3,5	2,3
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	15,1	6,8
STIMULACIJA CELIC Z IL-2 IN PHA		
Delež celic CD107a med celicami NK (CD56)	0,9	10,0
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	51,1	46,2
STIMULACIJA CELIC S CD3 IN CD28		
Delež celic CD107a med celicami NK (CD56)	2,2	7,0
Delež celic CD107a med citotoksičnimi limfociti T (CD8)	49,4	60,7

Etiology?

5.12. 2017

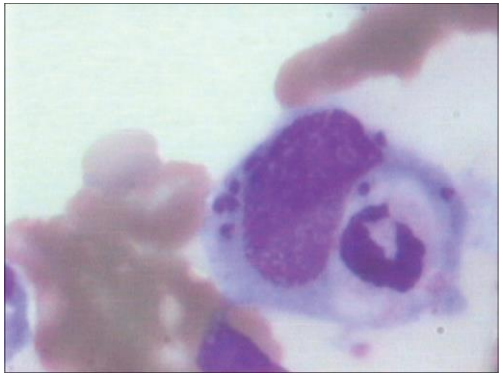
BM biopsy positive for *Leishmania spp*.; PCR positive for *Leishmania spp.*

Th: Medrol (metilprednizolon)

+

AmBisome (liposomal amfotericin B)

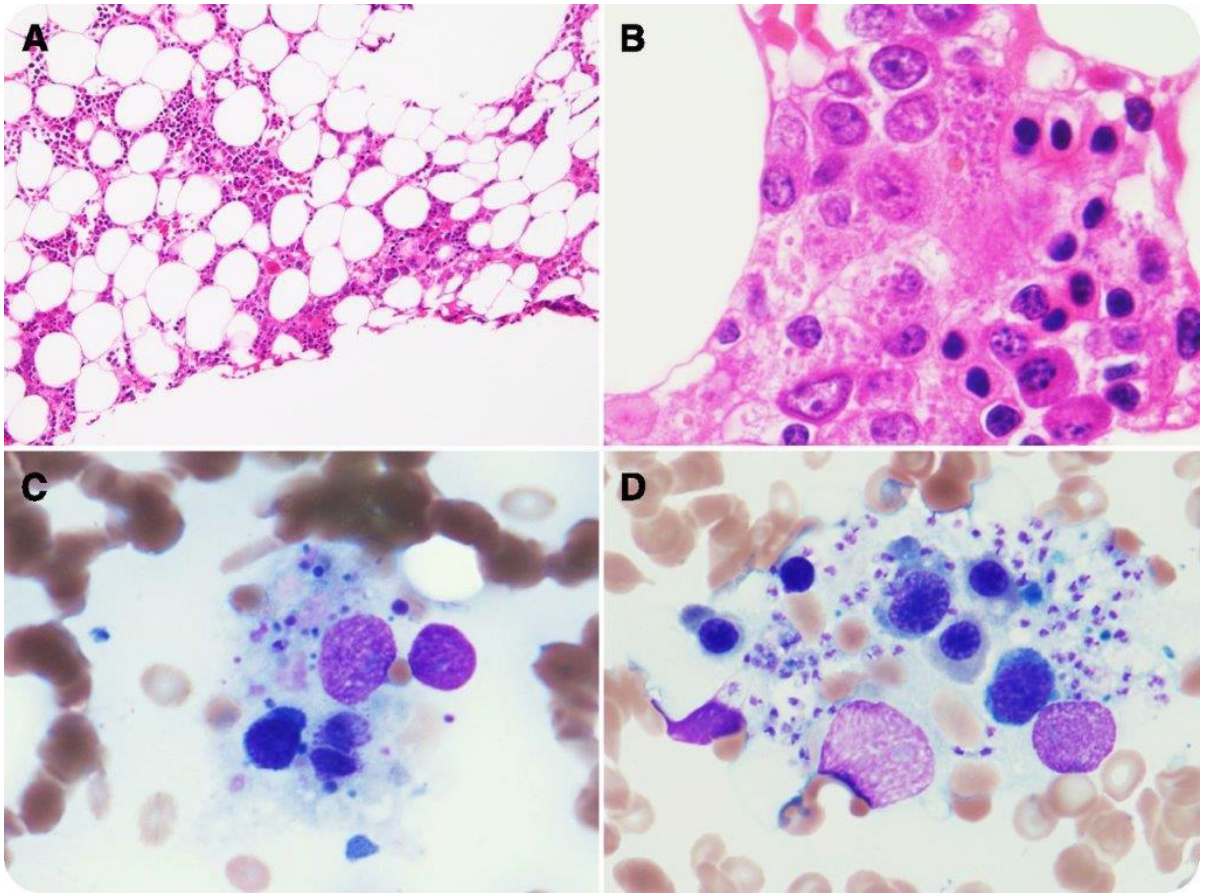
Significant improvement noticed, fever subsided,



Diagnostic Reasoning

- According to the guidelines set by HLH-2004, he fit the diagnostic criteria for HLH even without having hematophagocytosis in the bone marrow.
- He fulfilled at least 5 of the criteria with
  - Fever
  - Splenomegaly
  - Hypertriglyceridemia
  - Ferritin greater than 10000 microgram/L
  - Soluble CD25 (soluble IL-2 receptor) greater than 2400 U/ml

Hemophagocytic lymphohistiocytosis associated with visceral leishmaniasis



Matnani R. Blood 2016 127:513; doi: https://doi.org/10.1182/blood-2015-10-678862

A case report of recurrent fulminant myocarditis treated with a second ECMO run in a child

- A case of a 7-year old girl with two episodes of fulminant heart failure, both requiring cardiopulmonary resuscitation and extracorporeal membrane oxygenation support. Both episodes were caused by fulminant myocarditis that was precipitated by a viralupper respiratory tract infection.
- There was complete recovery of cardiac function following both episodes. The second episode was particularly complex with markedly elevated markers of myocardiocytolysis, multiorgan dysfunction and the need for prolonged mechanical circulatory support. Nevertheless, the patient made a remarkable recovery.
- A comprehensive diagnostic workup pointed towards an aberrant immune response as the likely cause of the girl’s susceptibility for fulminant myocarditis.

First episode of fulminant heart failure		
Age	5 years	
ECMO duration	82h	
Adverse events	Pleural effusion	
Blood biochemistry	Lactate	7.2 mmol/L*
	Troponin I	7.8 mcg/L*
	NT pro-BNP	22763 ng/L*
	AST	4.8 mckat/L*
	ALT	1.9 mckat/L*
Microbiology	Positive nasopharyngeal swab for parainfluenza virus	
	Positive serology for coxsackievirus	
Immunology	Reduced levels of CD3, CD4, CD8, CD19 lymphocytes and NK cells	
	Increased level of sIL-2R	
Genetics	Negative molecular karyotyping	
Metabolic	Significantly elevated urine ketones	
Other	Normal levels of lead, galactose, thyroid hormones and TSH, elevated levels of vitamin A and anti-TPO antibodies, negative screening for mucopolysaccharide disorders, normal Wood lamp examination	

Second episode of fulminant heart failure		
Age	7 years	
ECMO duration	373h	
Adverse events	Intracranial haemorrhage, partial amputations of lower extremities, liver damage, ARF, rhabdomyolysis, bloody aspirations from endotracheal tube, noncardiogenic pulmonary oedema, pleural effusion, osteoporosis, muscular atrophy, cachexia, gallstone, impaired healing of wounds	
Blood biochemistry	Lactate	19.8 mmol/L*
	Troponin I	229.3 mcg/L*
	CK MB	beyond 300 mcg/L*
	NT pro-BNP	beyond 35000 ng/L*
	AST	33.9 mckat/L*
	ALT	6.5 mckat/L*
	Total bilirubin	390 mcmol/L*
	Direct bilirubin	274 mcmol/L*
	Myoglobin	112129.9 mcg/L*
	CK	1258.7 mckat/L*
Microbiology	Positive nasopharyngeal swab and tracheal aspirate for influenza A virus (H3N2)	
Immunology	ANA	1:640
	MPO ANCA	37 U/mL, later negative
	Transiently reduced levels of CD3, CD4 and CD8, and persistently reduced levels of CD19 lymphocytes and NK cells	
	Increased ratio of gamma delta DNT among CD3 lymphocytes up to 17%	
	Normal complement activation for classic, alternative and lectin pathways	
	IL-6 cytokine response to TLR1-3 and 5-8 stimulation	

Initial tests of TLR function pointed towards an aberrant immune response, but further examinations will be required to determine with certainty the cause of the girl's susceptibility for recurrent fulminant heart failure during certain upper respiratory tract infections.

Circulation Research. 2016;118:496-514

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics

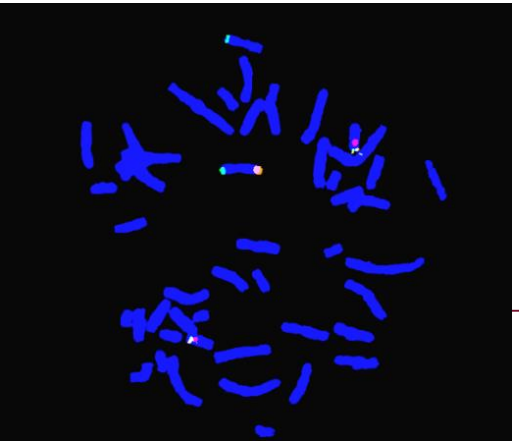
11q Terminal Deletion and Combined Immunodeficiency (Jacobsen Syndrome): Case Report and Literature Review on Immunodeficiency in Jacobsen Syndrome

Štefan Blazina,[1]* Alojz Ihan,[2] Luca Lovrečić,[3] and Tinka Hovnik[4]

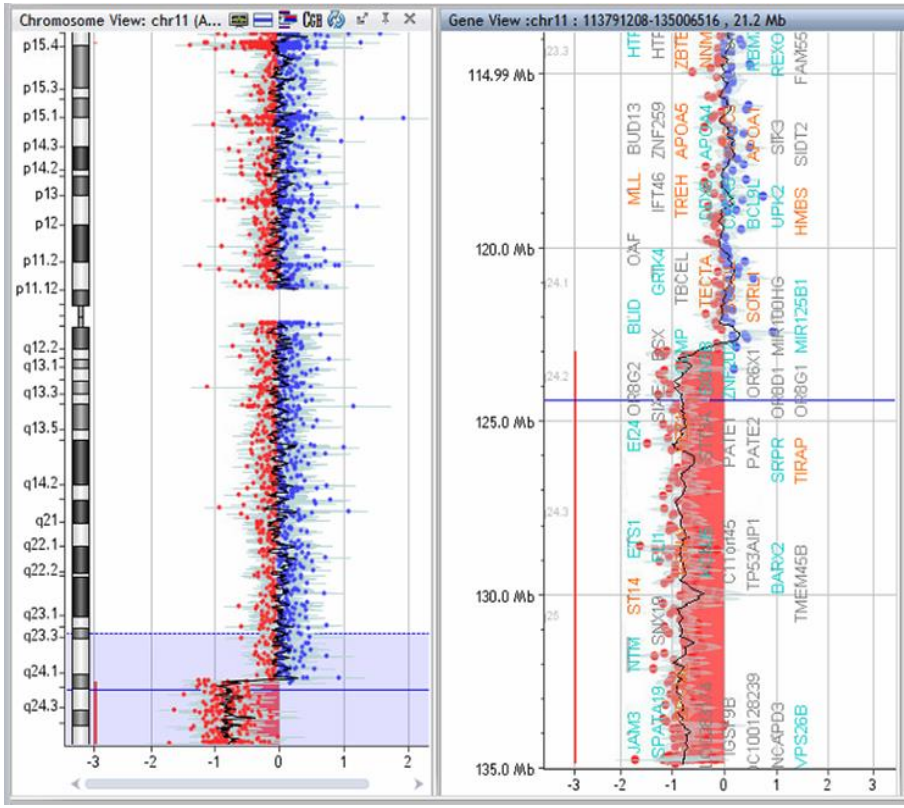
TABLE I. Lymphocyte Populations, Proliferation Response, Immunoglobulins, and Platelets Over Time

Age (years)	3	Normal values (3 year)	14	16	17	Normal values
T-cell (CD3+; $\times 10^6$/mm^3)	3555	1400–3600	745	751	742	700–1900
helper T-cell (CD3+ CD4+; $\times 10^6$/mm^3)	1712	700–2000	364	312	339	400–1300
cytotoxic T-cell (CD3+ CD8+; $\times 10^6$/mm^3)	1756	500–1400	345	402	347	200–700
naive T-cell (CD3+CD4+CD45+RA+; $\times 10^6$/mm^3)		430–1500	145	106	95	230–770
memory T-cell (CD3+CD4+CD45+RA-; $\times 10^6$/mm^3)		220–660	218	206	244	240–700
RTE (CD3+CD4+CD45+RA+CD31+; $\times 10^6$/mm^3)		50–926		47	41	50–926
Regulatory T-cell (CD3+ CD4+CD25++; %)		1–5			7	1–5
NK-cell (CD16+CD56+; $\times 10^6$/mm^3)	527	100–700	149	192	178	100–400
B-cell (CD19+; $\times 10^6$/mm^3)	351	400–1500	43	34	40	100–400
CD3 stimulation with PHA (%)		42–63		25	43	42–63
CD3 stimulation with anti-CD3 and anti-CD28 (%)		50–85			80	50–85
IgG (g/L)	6.4	4.70–12.30	6.24	8.91	7.38	6.90–14.00
IgA (g/L)	0.4	0.21–1.45	0.32	0.53	0.41	0.7–4.10
IgM (g/L)	0.4	0.41–1.56	<0.16	<0.17	<0.17	0.30–2.40
Platelets ($\times 10^{12}$/L)	266	194–345	184	245	204	194–345

Our patient presented with recurrent bacterial and prolonged viral infections involving the respiratory system, as well as other classic features of the syndrome. In addition to low IgM, IgG4, and B-cells, also low recent thymic emigrants, helper and naı̈ve T-cells were found.



A terminal deletion of chromosome 11 with breakpoint approximately at 11q23 region, which is described as a cause of JS. The deletion was confirmed using specific FISH analysis and the combined karyotype was 46, XX,del(11)(q23.3).ish del(11)(q23.3)(D11S1037-



Array comparative genomic hybridization analysis on genomic DNA extracted from peripheral blood sample (Agilent 60K ISCA Oligo, Agilent Technologies, Santa Clara, CA).

JS: Deletion of genetic material from the long arm of chromosome 11; microcephaly, trigonocephaly, hypertelorism, ptosis, epicantus

INDEX patient: girl, Mirjana B born 15.06.1991

Presentation
at 14d bloody diarrhea and trombocytopenia

In first 6m:
eczema
food allergy
infections
- skin Staph. Aureus
- UTI Proteus, Klebsiella
- GIT Enterobacter, Klebsiella
- RT CMV interstitial pneumonia

Anal fistula Staph aureus

Immunology:

L 35,9
increased eo **1700**

IgG 13,4 IgA 0,98 IgM 2,52;

cIgE **1746→338**;
(poz prick tests cows milk,
sIgE cows milk 26→2,6)

LSubsets:
Tly 48% Bly 17%
CD4+**13**%(**433)** CD8+19%(633)

TTL: **ConA↓**, PHA norm.

NBT 0,18; kemilum. norm.

Clinical picture:

INFECTIONS

RT - reccurent bronchiolitisis in preschool years
- 3x pneumonia (mostly preschool years)
- reccurent URT infections

ENT - Chronic sinusitis (H.inf., Corynebact.diph.,Strep.pn.)
- occasional OMA (E.fecalis, k- Staph) -

Skin - reccurent skin abscesses (monthly for several years)
(mostly S.aureus; also Corynebacterium, Difteroidies)

ALLERGY

eczema
in preschool years to food allergens (milk, egg, wheat)
later to pollens, mite and several medications (antibiotics – SMX)

AUTOIMMUNITY

AI trombocytopenia
nonspecific vasculitis – HSP like ; worse with infections
(3x skin biopsy :
(small granular and linear IgA on basal membrane of sweat glands, small granular IgM deposits on dermo-epidermal border, IgG neg., C3 small granular deposists, C1q+ cells in dermis)
panniculitis; worse with infections
increased inflammatory markers, increased SAA

Immunology

	CD3	CD4+	CD8+	CD19	NK
1991	**48%**	**13%**	19%	17%	
1999	**626**	**305**	498	562	369
2003		**278**	278		
2008		**192**	115		
2010	563	260	314	268	
2016					

	IgE	IgG	IgA	IgM
1991	**1746**	13,4	0,98	2,52
1998	**356**	12,8	**10,4**	1,0
2003	**780**	10,1	**7,6**	0,5
2008	**4461**	15,2	**11,40**	0,85
2010		12,5	**8,78**	1,49

Normal T cell transformation test (PHA, ConA) **Normal vaccination Ab**

- **Anemia**
- **thrombocytopenia**
- **petechiae, blood diarrhoea**
- **Infections, skin abscesses**
- **↑IgE ↑IgA**
- **eczema**
- **AI phenomens**

In 2016 gave birth of a healthy girl

Genetic tests:
46XX
Normal lymphocyte apoptosis

adenozin deaminaza neg.
Negative ALPS genetics (FAS –R, FAS –L) Negative HiperIgE

gen.diagnostics WAS (Brescia) -2005:
no mutation found

2nd patient: boy, Dario B born 30.11.1993

Presentation
at 4w bloody diarrhea

In first 6m:
enterocolitis
severe food allergy - parenteral feeding

infections
- skin asbcesses (Staph. Aureus)
- perianal abscess
- RT pneumonia

Failure to thrive

Immunology:

L 12,3, Tr 168

IgG 11,3 IgA 4,7 IgM 0,88;

cIgE 270→932

LSubsets:
Tly 44% Bly 34%
CD4+13% CD8+34%

TTL: ConA and PHA↓

Clinical picture:

INFECTIONS

RT - several prolonged pneumonias
(Morax. catarrh., Strep.pneum., Corynebact., C. albicans)
- reccurent URT infections

GIT - gastroenteritis
(Klebsiella, Pseudomonas, Cryptosporidium, Bacillus cereus, Enterobacter agglomerans,Citrobacter freundii, ncephalitozoon intestinalis)
- Candida Esophagitis

Sepsis – with central line (Staph aureus, Strep. Viridans)

Skin - reccurent skin abscesses in last years

ALLERGY

food allergens - prolonged nad slow introduction of foods

AUTOIMMUNITY

nonspecific vasculitis ; worse with infections

Pernicious anemia – clinical picture of vitB12 deficiency (delayed motoric development, CNS atrophy with acute neurological simtoms)

stunted growth - on therapy with GF

TREATMENT:

No prophylaxis (occasional Septrin - TMP7SMX

Vit B12 supplementation

Immunology

	IgE	IgG	IgA	IgM
1997	**932**	11,3	**4,70**	0,85
2001	**257**	9,9	**5,6**	1,3
2006	**441**	9,70	**7,33**	0,30
2010	/	9,55	**4,47**	0,18
2016		9,71	**4,57**	<0,19

	CD3	CD19	CD4+	CD8+	NK
1997	44%	34%	**13%**	34%	20%
1999	2171	2714	**882**	**1696**	1696
2001			**459**	**546**	
2003			**327**	**358**	
2010			464	560	
2016	999	531	606	382	195

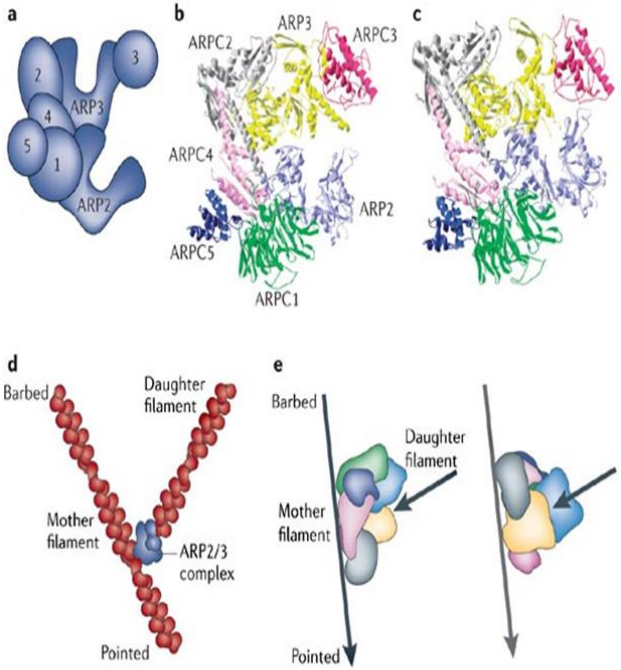
NBT 0,54,

normal / increased DHR

On NGS – mutation in one of the ARP2/3 subunits

=ARPC1B gene

NM_005720.3: c.265A>C **NP_005711.1: p.Thr89Pro**



ARP 2/3 complex:

Arp2 in Arp3 (Arp3 in Arp3β)
ARPC2 in ARPC4

ARPC3, ARPC5
ARPC1 (ARPC1A in ARPC1B)

3rd patient: girl, Miša K, born 20.5.1989

Presentation
- in first weeks with eczema and bloody diarhea

In first year:
- prolonged hospitalization (4y)
- enterocolitis
- severe food allergy

- Failure to thrive

Clinical picture:

INFECTIONS

RT - reccurent pneumonias since 3y (2-3 x/y) last in 2015
- lung abscess

ENT - reccurent sinusitis and OMA with perforation

GIT - gastroenteritis

Skin - reccurent skin abscesses sicnce age 10;
- chronic leg ulceration (last 2y – Strep. Constelatus)

Genital condiloma nad severe warts (last 5y)

ALLERGY

food allergens - prolonged nad slow introduction of foods
allrgy to mites and animal epithel
Alergic astma

AUTOIMMUNITY

enterocolitis with complications
- duodenum ulceration in 2007 – surgical intervention
- prepiloric deformation with suspected stenosis
- rectovaginal fistula

nonspecific IgA vasculitis ; worse with infections
stunted growth on therapy with GF
suspected polinevropathy (norm. B12, low folic acid)

(PRE)MALIGNANCY

Conisation of uteral nect (CIN2 lesions)

TREATMENT:

No prophylaxis
Therapy for astma
Ocasional metilprednisolon (for vasculitic exacerbations)

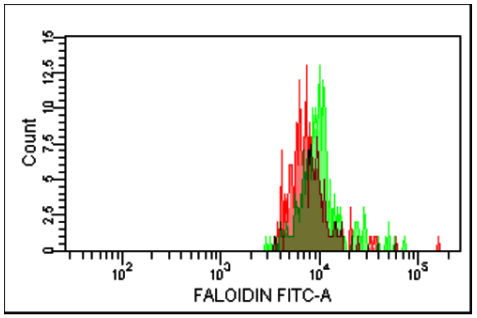
Immunology

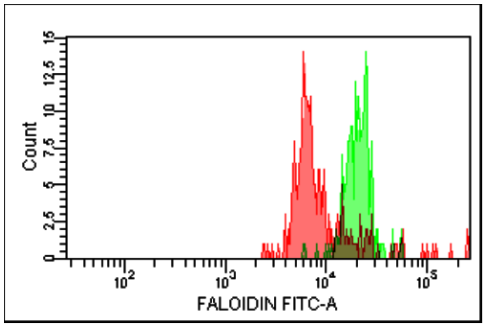
	IgE	IgG	IgA	IgM
2016	**716**	**13,6**	**6,3**	0,6
2017	**524**	8,4	**3,8**	0,4

	CD3	CD19	CD4+	CD8+	NK
2016	1006	542	730	226	**66**
2017	696	375	541	**122**	97

No naive T Cells
Decreased fagocitosis

Functional fMLP/phalloidin test





	Average MFI		
	before stimulation	**after stimulation**	**increase in MFI**
Neutrophils			
homozygous (n=9)	10.285 ± 2.883	11.408 ± 2.346	11%
carriers (n=4)	16.489 ± 9.752	26.308 ± 5.911	60%
without mutation (n=3)	8.893 ± 4.983	17.670 ± 7.519	99%
Monocytes			
homozygous (n=9)	3.518 ± 674	3.710 ± 507	5%
carriers (n=4)	4.892 ± 959	8.799 ± 1.455	80%
without mutation (n=3)	3.795 ± 2.278	7.862 ± 4.532	107%

Average median fluorescence intensity (MFI) with standard deviations of neutrophils and monocytes before and after 20 seconds of stimulation with fMLP in three different groups – homozygous (patients), carriers, and healthy subjects (without ARPC1B mutation).

Morphological Assay for Actin Polymerization

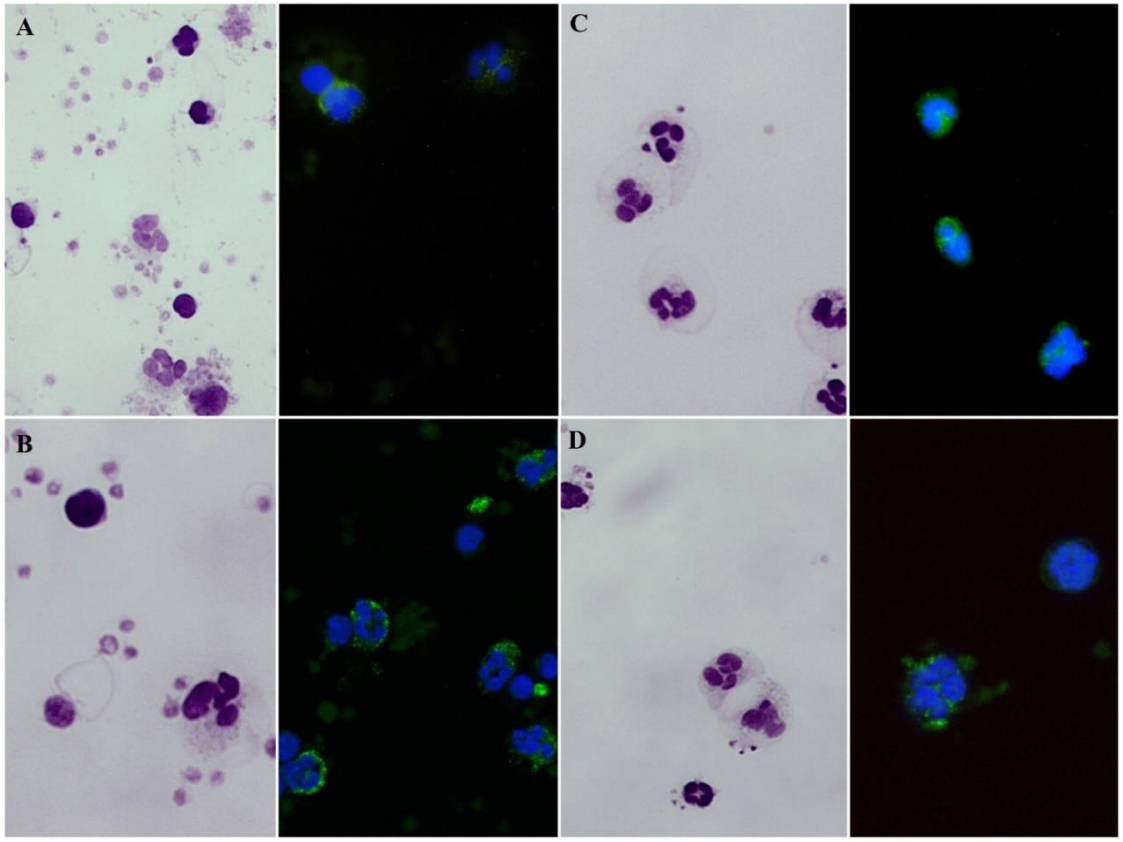


Figure 2. Giemsa stained blood smears and fluorescent microscopy images of FITC-phalloidin stained cells. A. Smears of non-stimulated blood specimen of a healthy subject. B. fMLP-stimulated blood cells of a healthy subject. C. Non-stimulated patient's blood cells. D. fMLP-stimulated patient's blood cells.

Slovenian PID team

Thank YOU!!

Questions??

SURFACE AND INTRACELLULAR FLOW CYTOMETRY

Immunophenotyping methods for staining surface and intracellular CD markers of peripheral blood and bone morrow are long established. As increasing number of intracellular antibodies becomes available there is a need to evaluate these antibodies for flow cytometry applications. Flow cytometry is a popular technology, it is rapid and sensitive. For example, the biomarkers of the CD34 population in a leukemia patient could be followed over the course drug treatment down to 0.01% CD34 positive in peripheral blood.

In clinical trial whole blood assay is essential to investigate and identify the status of the patient's signaling pathways and the pharmacodynamic monitoring of drug treatment over time which involve a multiple tube panel with stimulators and inhibitors. After extensive testing we have developed a simple and effective three component sample preparation method to detect surface CD markers and other intracellular antigens in whole blood and cell cultures: (1) fixation and (2) permeabilization (3) with or without 80% methanol treatment.

1. **Fixation:** formaldehyde

- 4% for whole blood
- 2% for cell culture
- 10 minutes at room temperature, these two parameters are critical and must be followed precisely.

Formaldehyde a cross-linking agent is used to fix cells in suspension to preserve and maintain structure and location of target eptiopes. For whole blood 4% formaldehyde preserves the forward scatter and side scatter resolutions of the white blood cell subpopulations and at the same time allows lysis of the red blood cells. Complete RBC lysis is important for flow cytometry as RBCs outnumber WBCs about 1000 to 1. **Formaldehyde fixation must be done at room temperature, at 37C RBCs would not lyse.** For cell lines 2% formaldehyde is usually enough. In the study of phospho-proteins formaldehyde fixes the phosphorylation of target amino acids (Serine, Threonine , and Tyrosine) and also inhibits the phosphatases which de-phosphorylate these targets rapidly in live cells.

2. **Permeabilization:** 0.1% Triton X-100

- 15 minutes at 37C

In order for antibodies to get inside the cells, formaldehyde fixed cells are permeabilized with 0.1% Triton X-100 for 15 minutes at 37C. Complete RBC lysis occurs for 99% of clinical blood samples with incubation at 37C for 15 minutes. Hence incubation at 37C became routine for cell lines as well. Incubation time is not as critical for the 0.1% Triton X-100 step but is kept at 15 minutes unless longer time is required for complete RBC lysis. If after 30 minutes of incubation there is still incomplete RBC lysis, the sample is spun down and fresh 0.1% Triton X-100 is added and incubation at 37C for up to another 30 minutes with monitoring.

Higher concentration of Triton X-100 e.g. 0.2%, will lyse RBCs faster but the WBCs become sticky and clumpy. On the other hand lower concentration, e.g. 0.05%, tend to give incomplete RBC lysis in 15 minutes.

3. 80% Methanol denaturation: 80% methanol in 0.9% (final concentration) NaCl

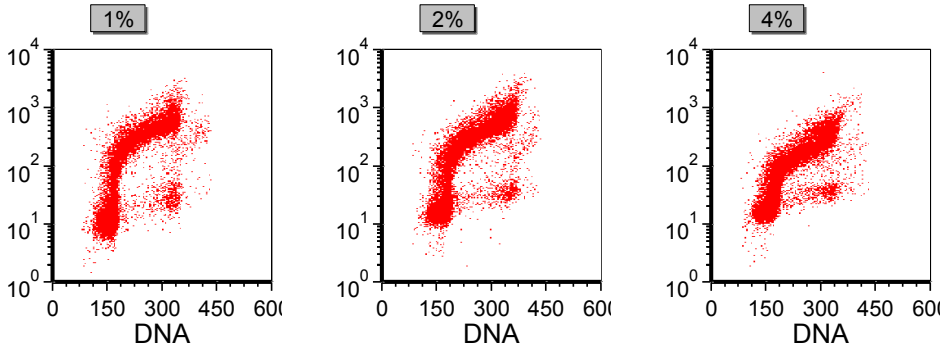
- 10 minutes on ice

After cross-linking fixation with formaldehyde, some epitopes, such as the phospho-STATs, require exposure to methanol for binding by the antibody. Although cells can be fixed and permeabilzed with methanol in one step, this is not recommended because without cross-linking fixation, small molecules of interest will leak out of the cells. **Very important, fixed and permed blood samples must undergo an extra wash to get rid of the hemaglobin from the RBC lysis before the 80% methanol treatment.** High methanol concentration and hemaglobin combination will destroy blood samples for antibody staining.

FORMALDEHYDE

Some CD markers (CD19, CD11b) and intracellular biomarkers are very sensitive to formaldehyde fixation. For CD markers, using a different clone or different CD marker may be the solution. Below are two examples of effect of formaldehyde concentration on two intracellular biomarkers: Cyclin A2 and Dimethylated Histone H3 Lysine 9.

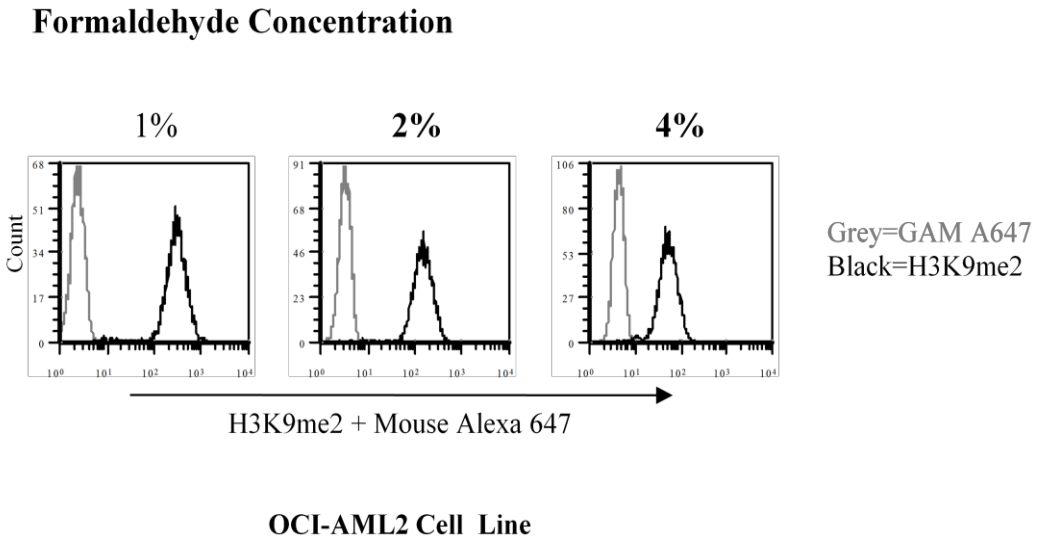
A. FORMALDEHYDE CONCENTRATION ON CYLIN A2 STAINING

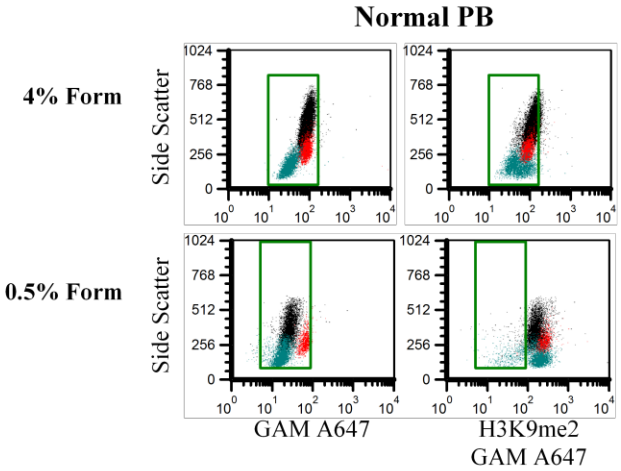


AML2 cell line, gated on single cells

B. FORMALDEHYDE FIXATION OPTIMIZATION for DIMETHYLATED HISTONE H3 (LYS 9)

First leukemia cell line OCI-AML2 was used, then normal blood in preparation for clinical samples.



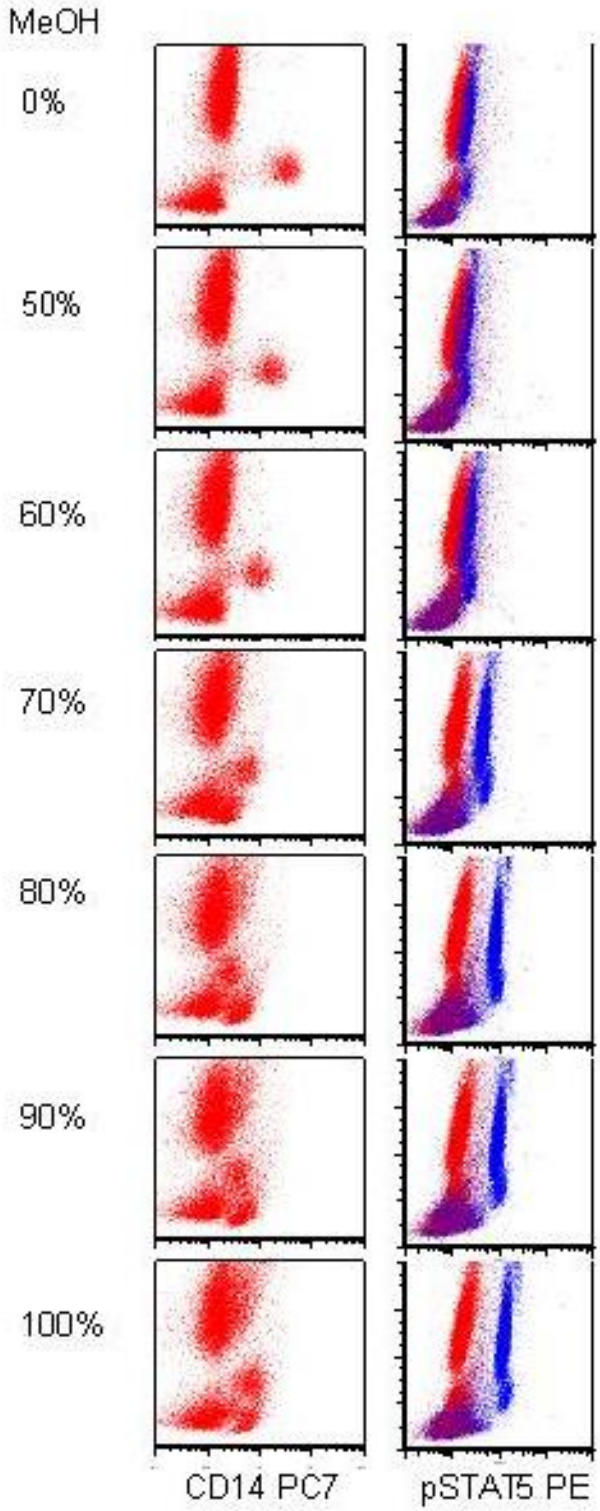


Four percent formaldehyde still gives useful signal in AML2 cells but not in normal blood. To use this H3K9me2 antibody in patient samples 0.5% formaldehyde is required.

Lesson learned: Using cell lines for method development is a good place to start but ultimately it is the model of interest that matters. Work on your model ASAP.

Important note: Despite several attempts we were unsuccessful in making a direct Alexa Fluor 647 conjugate of H3K9me2.

METHANOL TREATMENT



Effects of methanol on pSTAT5 staining and CD14 surface staining.

Normal donor blood was stimulated with GM-CSF, which activates JAK/STAT in granulocytes and monocytes, versus control, then fixed with 4% formaldehyde.

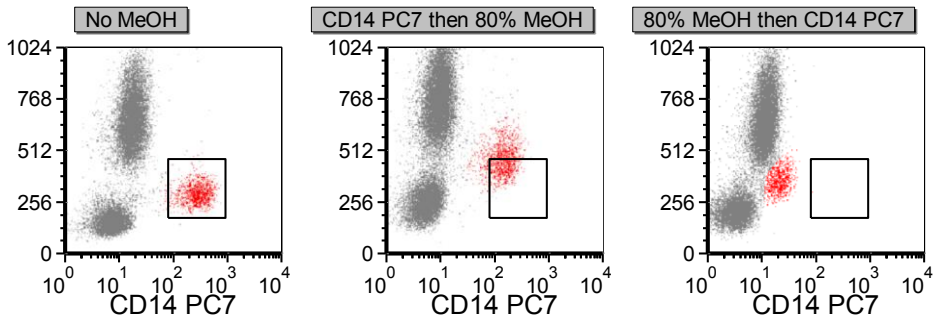
Control
GM-CSF

Vertical axis is side scatter for both sets of panels. Note that with 0% or 50% methanol there is excellent CD14 staining of the monocytes, but almost no pSTAT5 signal. With increasing methanol the pSTAT5 signal improves, but CD14 staining is lost.

Most surface markers are compromised to some degree with methanol. This is severe in the case of CD14, but other markers like CD34 and CD117 are better preserved.

Our standard protocol uses 80% as a compromise that gives good pSTAT5 while preserving most surface markers expressed by AML patients.

CD14 staining of normal blood after fix and perm



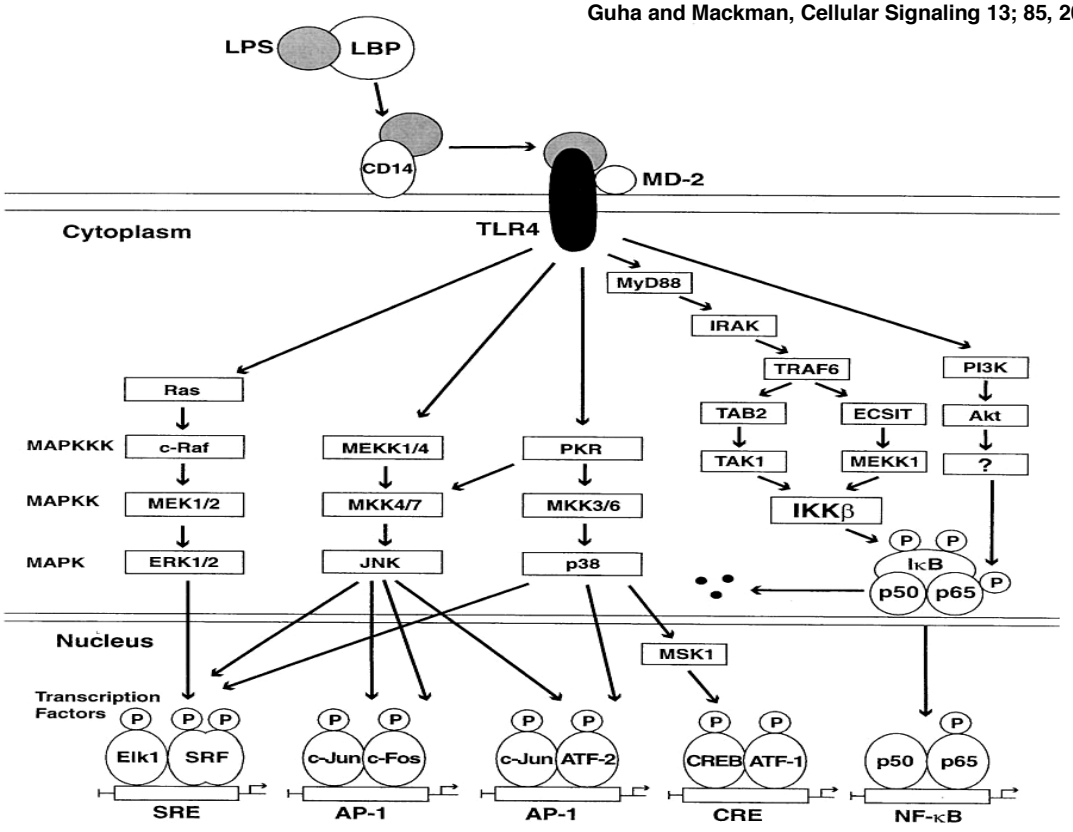
For antibody staining that required 80% methanol treatment, cells can be stained with the methanol sensitive CD marker(s) before the 80% methanol step. Alternatively, use a different clone or a different CD marker that is not affected by high methanol concentration to identify the subpopulation of interest e.g. CD33 instead of CD14 to gate on monocytes.

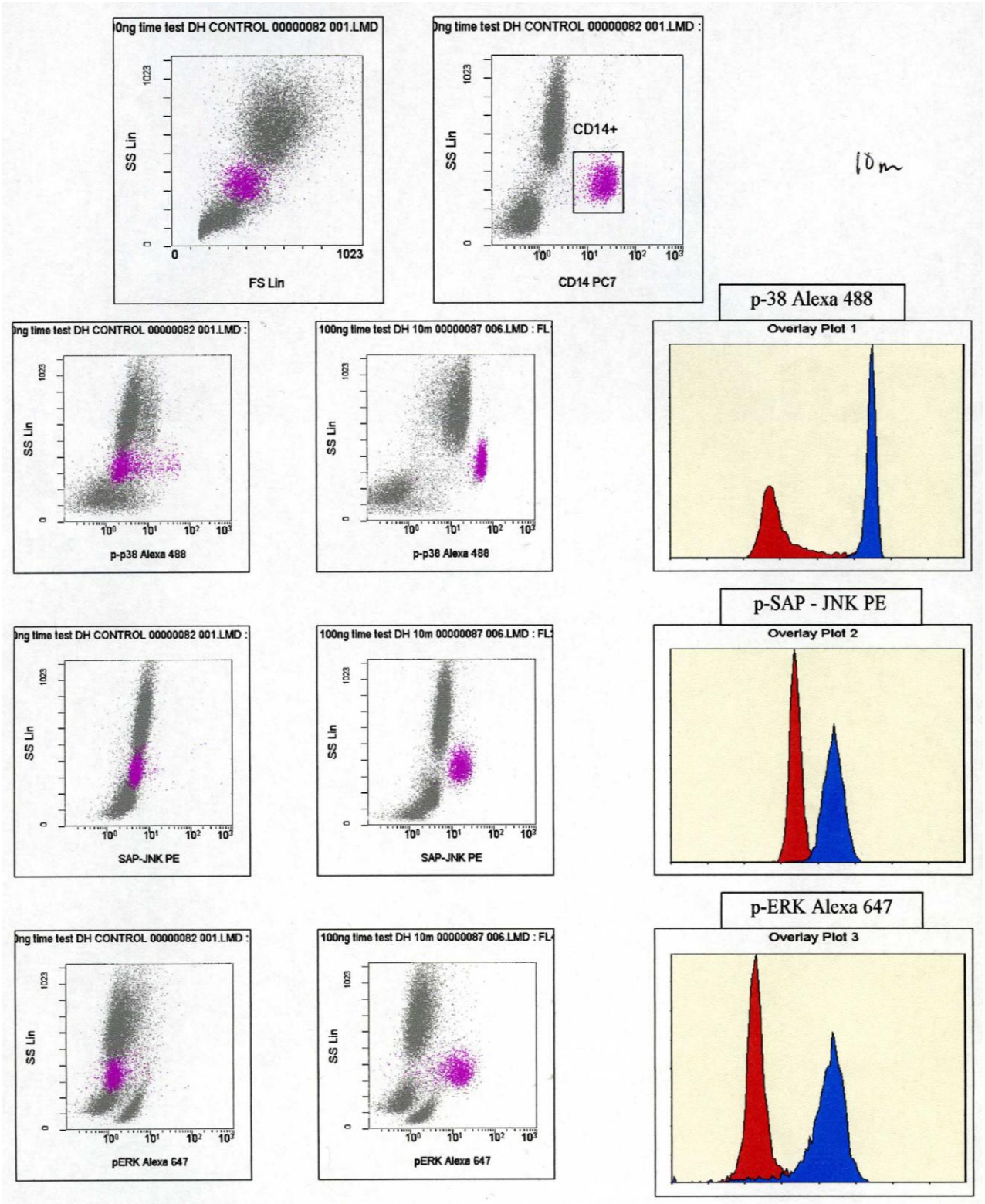
DOSE-TIME RESPONSE

Dose -Time Response assay is necessary to determine agonist concentration and stimulation time to achieve maximum signal. For whole blood incubation for 30 minutes at 37C is usually most informative. Longer incubation in a 37C bathe compromises the assay, the blood gets darker.

Time Response of LPS

Guha and Mackman, Cellular Signaling 13; 85, 2001

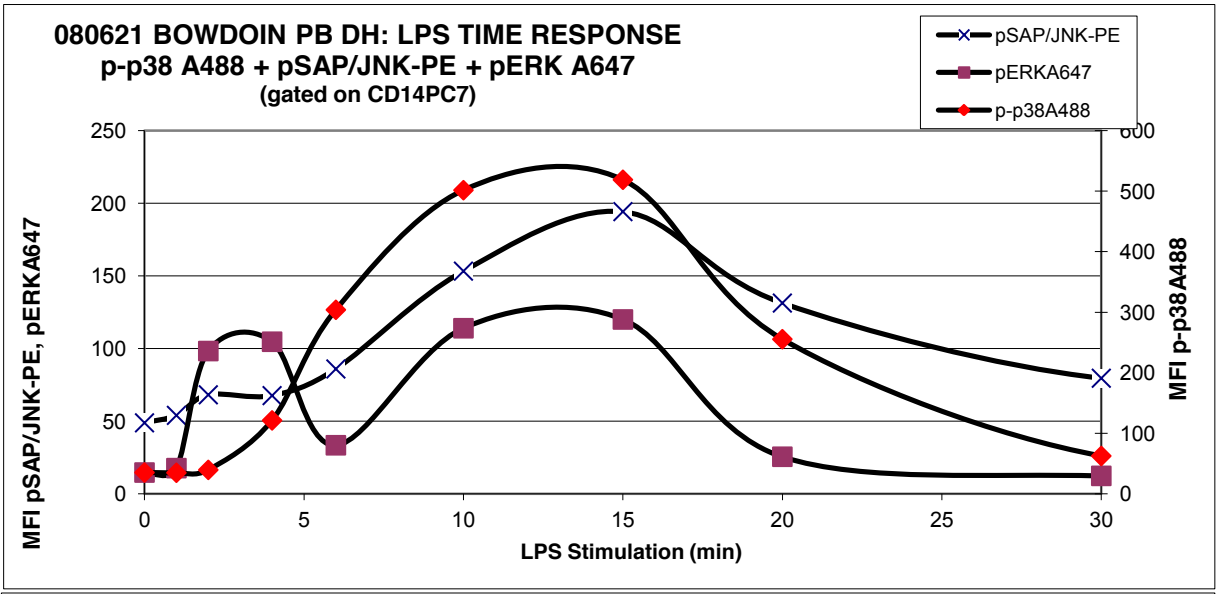


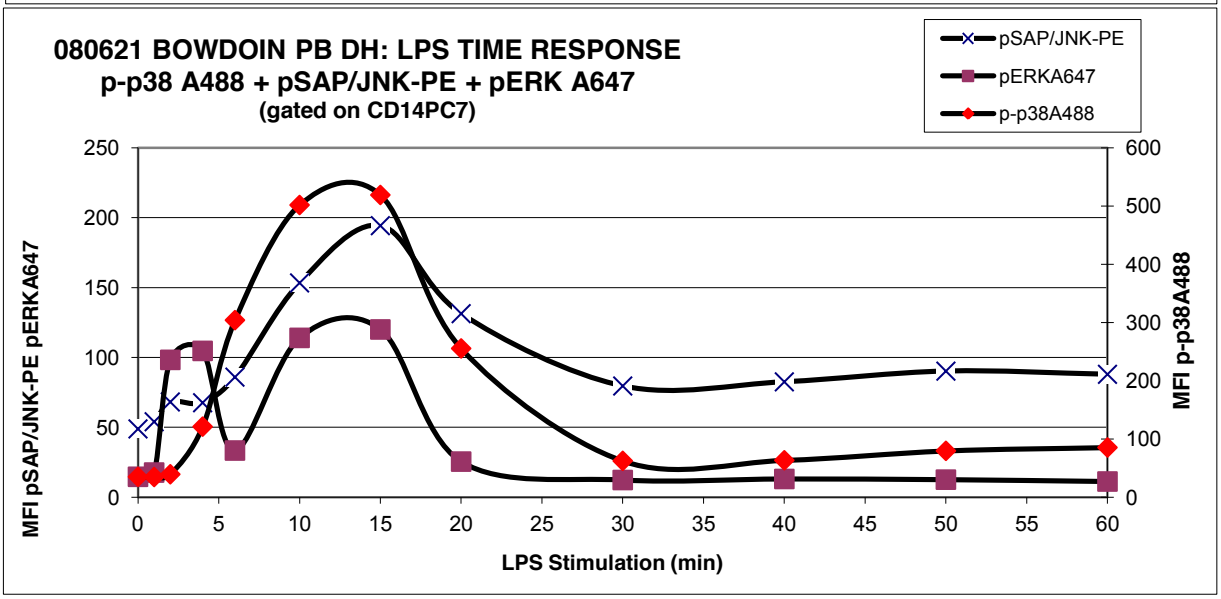


Lipopolysaccharide (LPS), bacterial endotoxin, 100ng/ 100uL blood, 10 minutes at 37C

080621 PB DH: LPS (100ng/ 100uL PB), p-p38A488 + pSAP/JNK-PE + pERKA647 30' stain RT

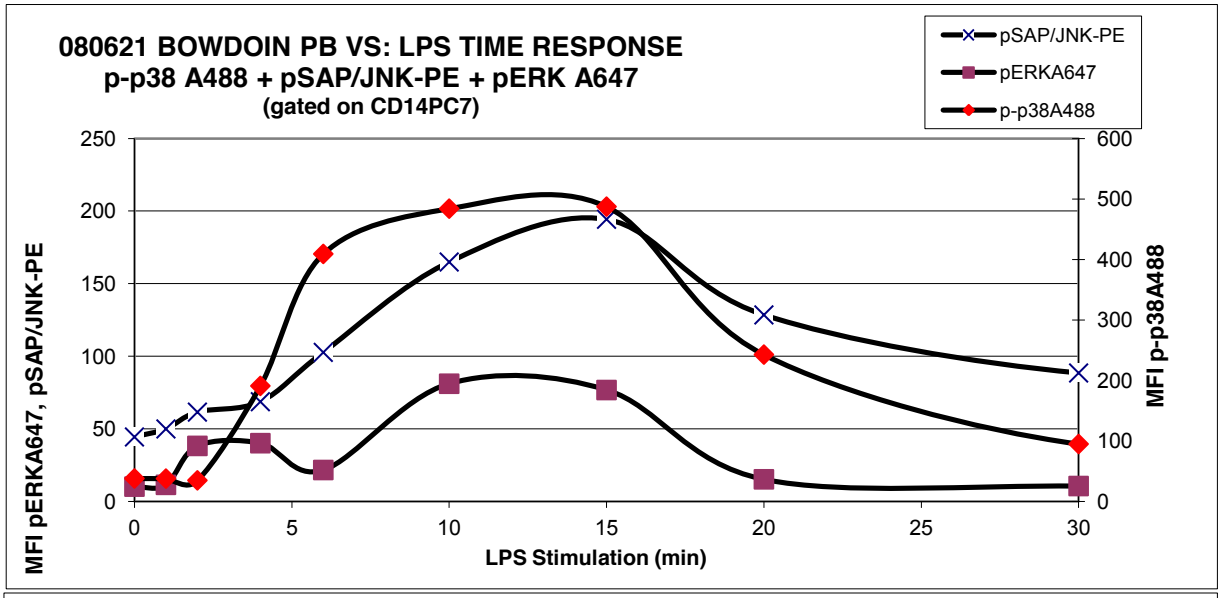
	Minutes	Filename	p-p38A488	SAPJNK-PE	pERKA647
Time points:	0	LPS 100ng time test DH CONTROL	35	48.82	14.58
	1	LPS 100ng time test DH 1m	34.64	53.96	17.62
	2	LPS 100ng time test DH 2m	39.36	68.11	98.25
	4	LPS 100ng time test DH 4m	121.14	67.56	104.65
	6	LPS 100ng time test DH 6m	303.96	85.91	33.37
	10	LPS 100ng time test DH 10m	501.68	153.29	113.96
	15	LPS 100ng time test DH 15m	518.91	194.26	120
	20	LPS 100ng time test DH 20m	255.38	131.3	25.45
	30	LPS 100ng time test DH 30m	62.38	79.54	12.28
	40	LPS 100ng time test DH40m	63.36	82.5	13.02
	50	LPS 100ng time test DH 50m	79.55	90.18	12.54
	60	LPS 100ng time test DH 60m	85.3	87.9	11.21

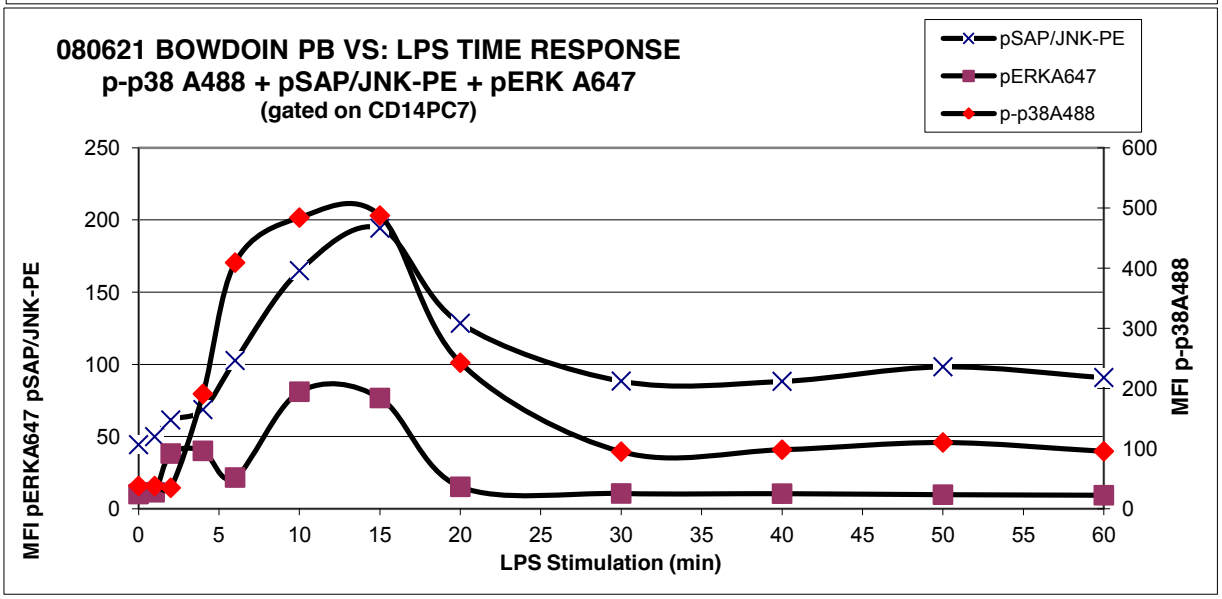




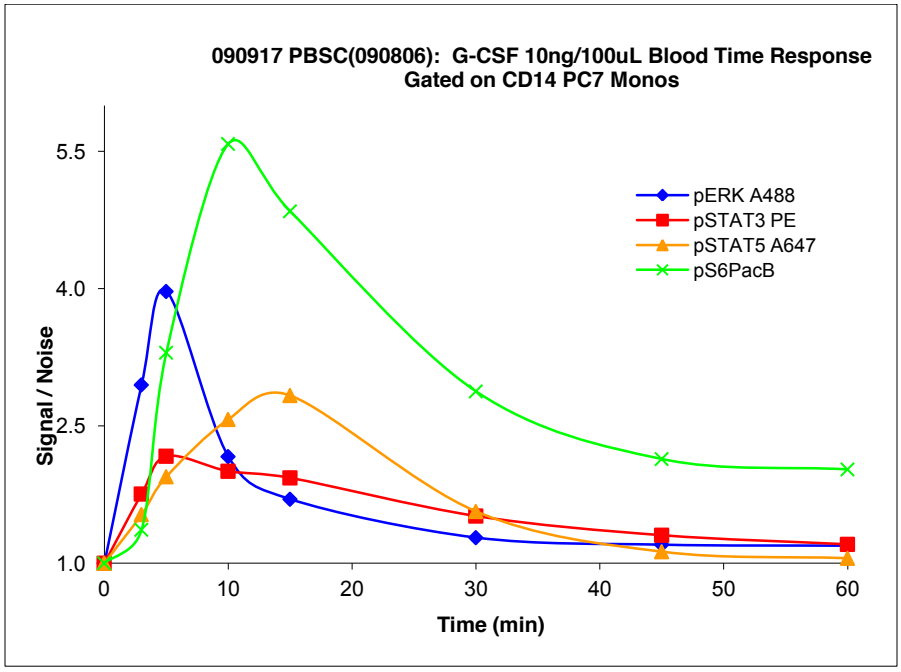
080621 PB VS: LPS (100ng/ 100uL PB), p-p38A488 + pSAP/JNK-PE + pERKA647 30' stain RT

	Minutes	Filename	p-p38A488	SAPJNK-PE	pERKA647
Time points:	0	LPS 100ng time test VS CONTROL	38.01	44.42	10.23
	1	LPS 100ng time test VS 1m	37.83	49.96	11.47
	2	LPS 100ng time test VS 2m	34.9	61.55	38.28
	4	LPS 100ng time test VS 4m	191.01	68.71	40.14
	6	LPS 100ng time test VS 6m	409.17	102.68	21.67
	10	LPS 100ng time test VS 10m	483.99	164.92	81.08
	15	LPS 100ng time test VS 15m	487.33	194.33	76.74
	20	LPS 100ng time test VS 20m	242.77	128.48	15.26
	30	LPS 100ng time test VS 30m	94.99	88.48	10.64
	40	LPS 100ng time test VS 40m	98.26	88.27	10.5
	50	LPS 100ng time test VS 50m	110.24	98.35	9.81
	60	LPS 100ng time test VS 60m	95.63	90.91	9.42



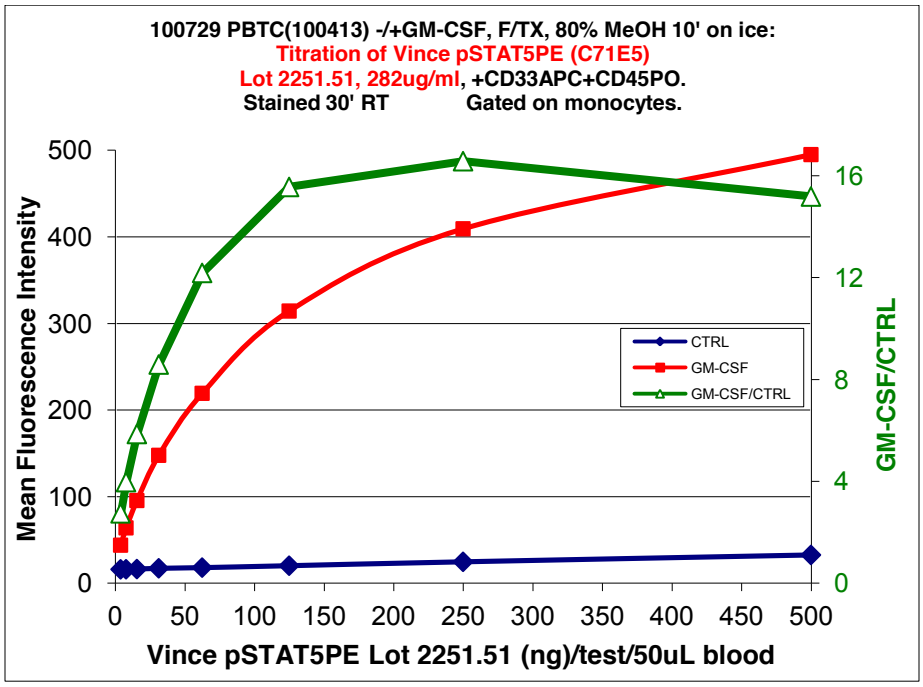


Time Response of G-CSF

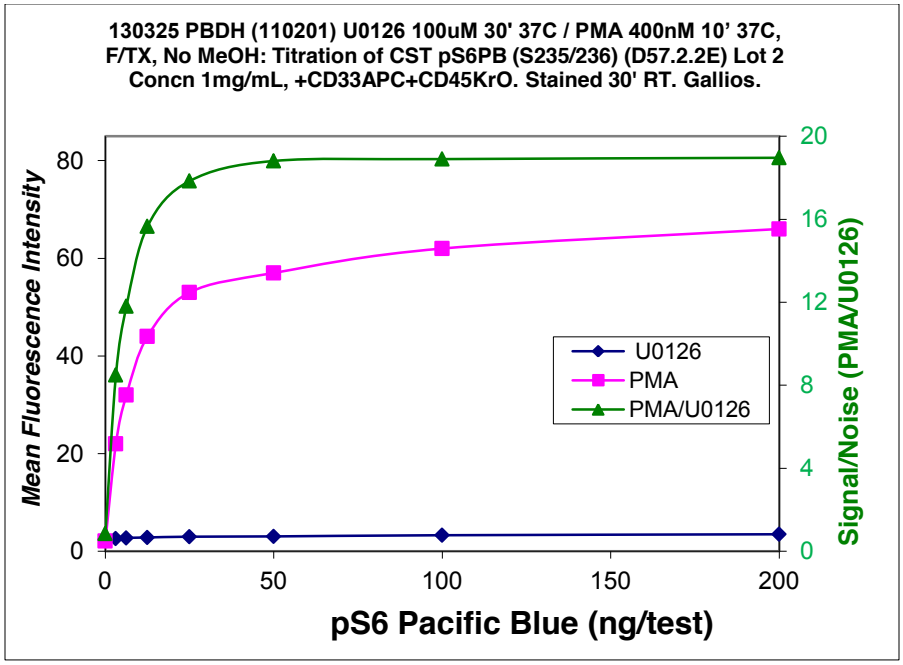


ANTIBODY TITRATION

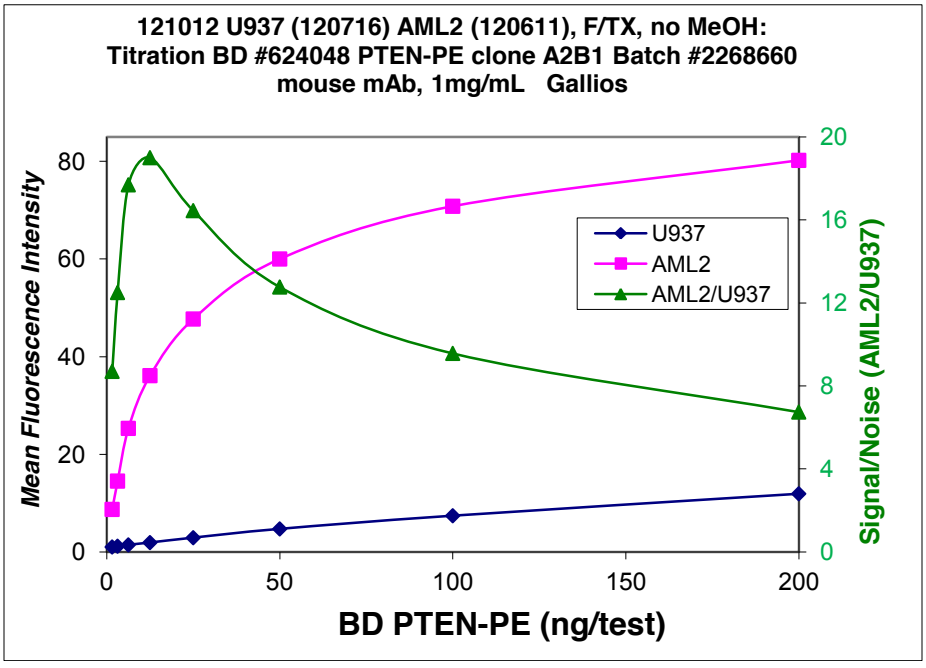
I. **Use a strong agonist to obtain largest signal separation between stimulated and untreated**



II. **If a pathway is constitutively active, use an inhibitor to decrease the signal to background**

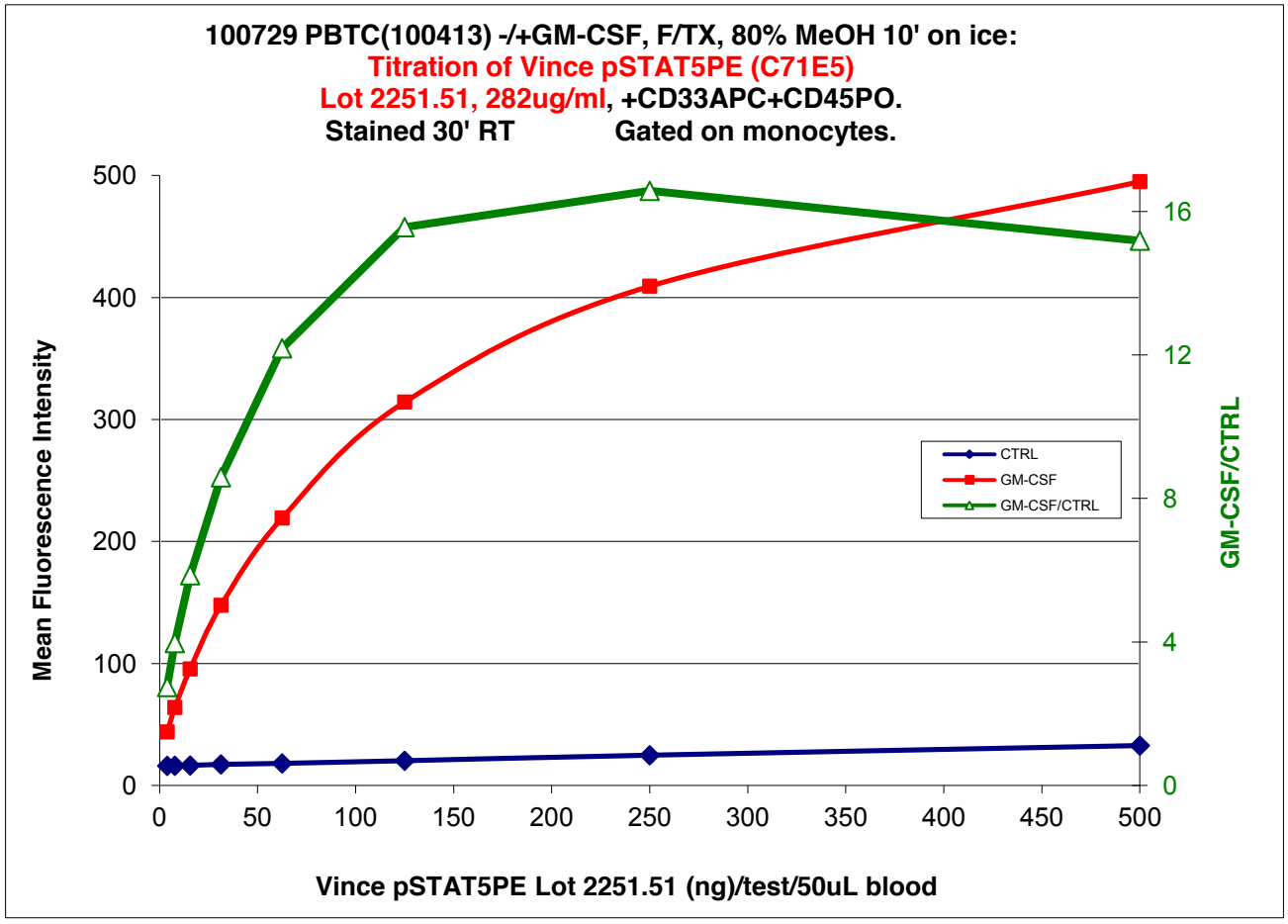


III. **If cannot stimulate cells to generate positive signal, use a low expresser and a high expresser of the signal of interest**

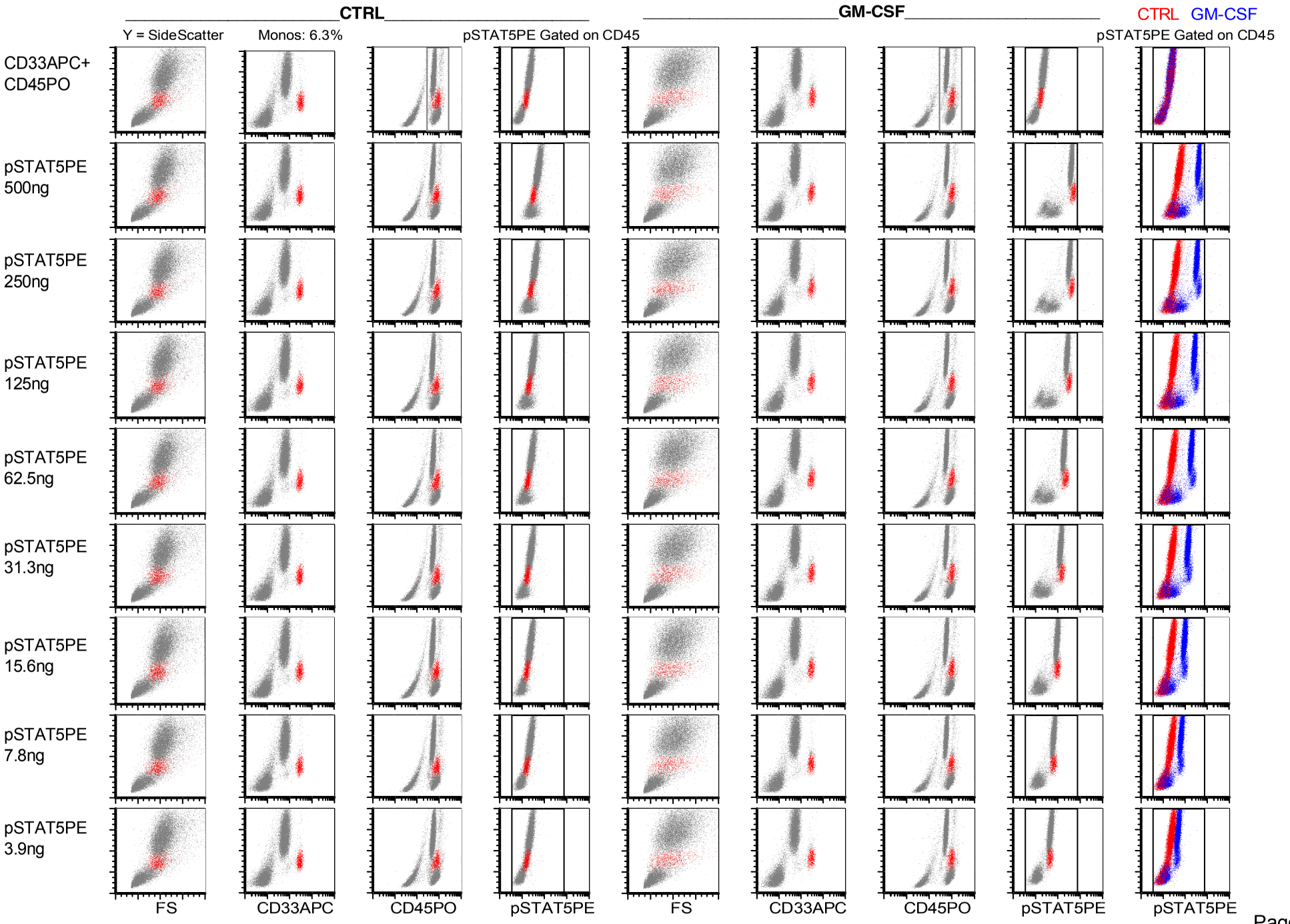


100729 PBTC(100413) -/+ GM-CSF 10ng/100uL, 10' 37C, F/TX, 80% MeOH 10' on ice:
Titration of Vince pSTAT5PE (C71E5)
Lot 2251.51, 282ug/ml, +CD33APC+CD45PO.
All staining 30' RT, run on Gallios.

	pSTAT5 (ng)	CTRL	GM-CSF	GM-CSF/CTRL	
1	500	32.6	495.0	15.2	Gated on Monos
2	250	24.7	409.3	16.6	
3	125	20.2	314.3	15.6	
4	62.5	18.0	219.3	12.2	
5	31.3	17.2	147.7	8.6	
6	15.6	16.3	95.5	5.9	
7	7.8	16.1	63.9	4.0	
8	3.9	16.0	43.9	2.7	
9	CD33APC+CD45PO	15.3	16.9	1.1	



100729 PBTC(100413) -/+GM-CSF 10ng/100uL, F/TX, 80% MeOH 10' on ice: Titration of Vince pSTAT5PE (C71E5)
Lot 2251.51, 282ug/ml, +CD33APC+CD45PO. All staining 30' RT. Gallios.



ANTIBODY EVALUATION for FLOW

Same procedure is used for both primary and conjugated antibodies.

1. Usually need a negative and positive pair of cells. Exceptions include: Cyclin A2, Cyclin B1, and phospho-histone Ser10.

2. Fix and Perm: formaldehyde (4% for blood, 2% for cell lines) and 0.1% Triton X-100.

3. With and without 80% methanol treatment.

4. If above conditions give poor antibody staining, check formaldehyde sensitivity by titration of formaldehyde concentration (0.5, 1, 2, and 4%).

5. Antibody titration to determine amount of antibody to use per test.

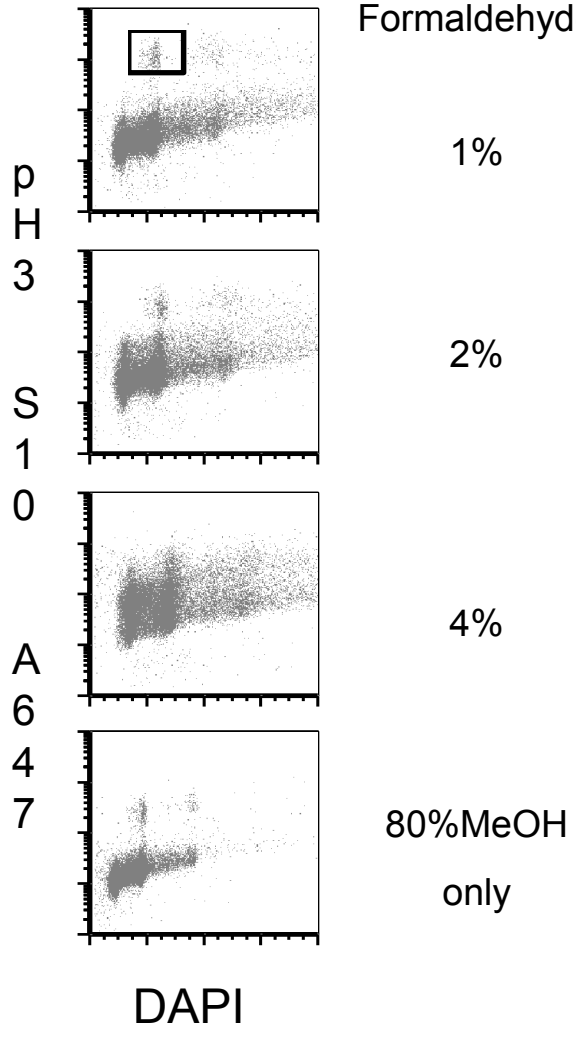
6. If needed large batch of negative and positive cells can be prepared and frozen at -20C for future use; such as, new antibody titration and positive staining control.

Antibody Evaluation 1

BioLegend asked me to try their phospho-H3 ser 10 antibody.

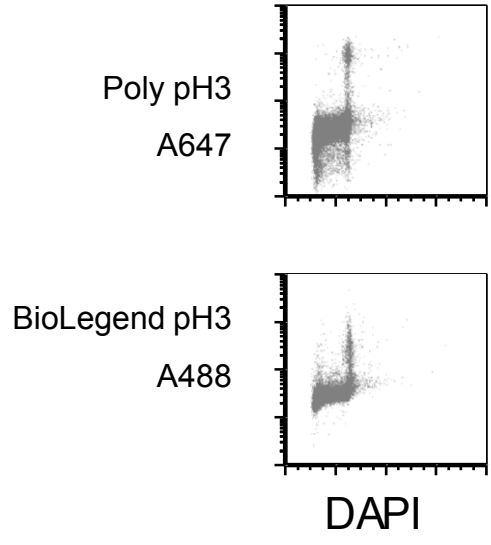
Previous Experiment

Effect of formaldehyde concentration on pH3 S10 (polyclonal-A647) staining in AML2 cell line

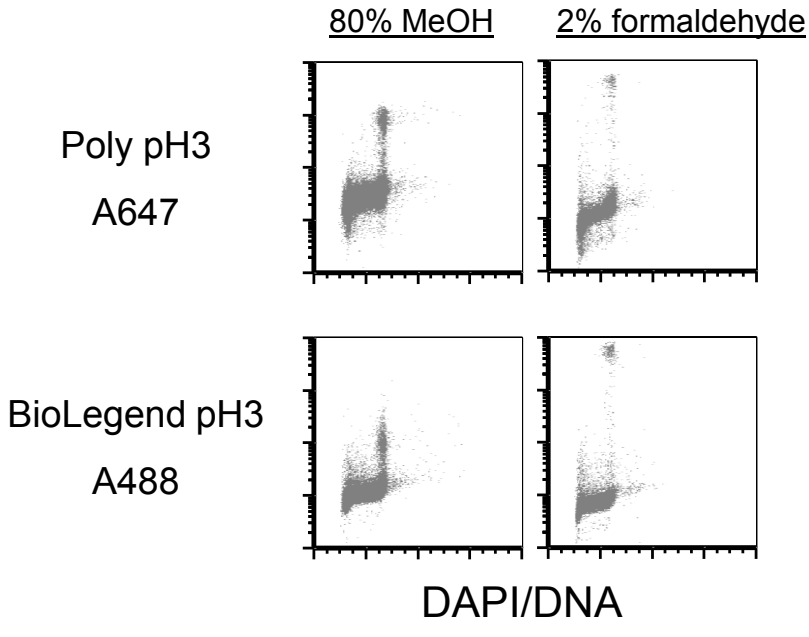


Results: Increase concentration of formaldehyde decreased pH3 S10 staining and 80% methanol treated AML2 cells gave good pH3 S10 staining.

Based on these results I decided to use 80% MeOH treated AML2 cells to test BioLegend pH3 S10 A488 (Cat no. 650803)

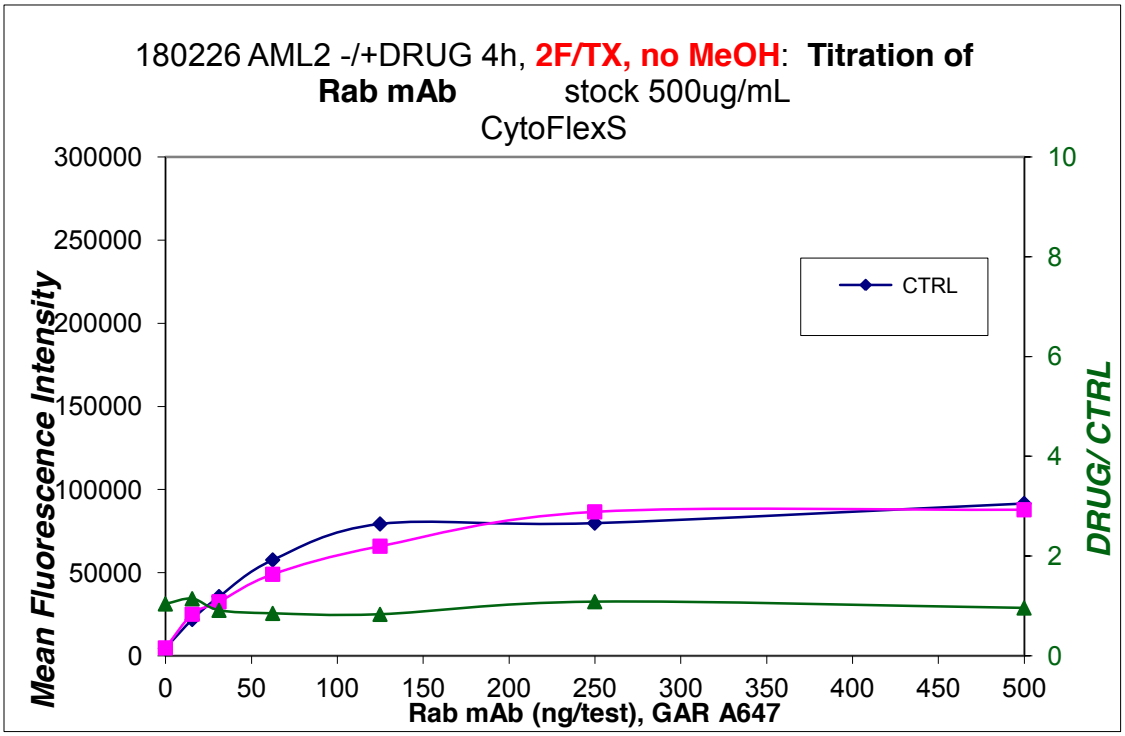


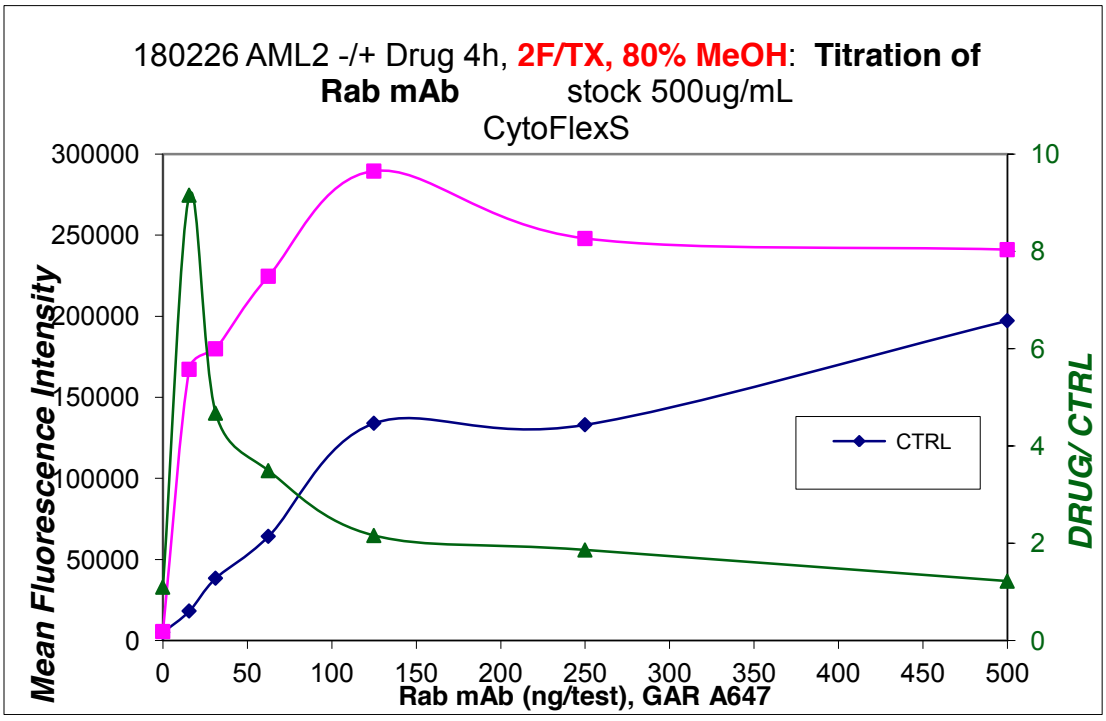
I was disappointed with these results but decided to compare staining in 2% formaldehyde fixed vs 80% methanol treated AML2 cells. These are one batch of cells fixed and permed in the different ways and froze down the same day.

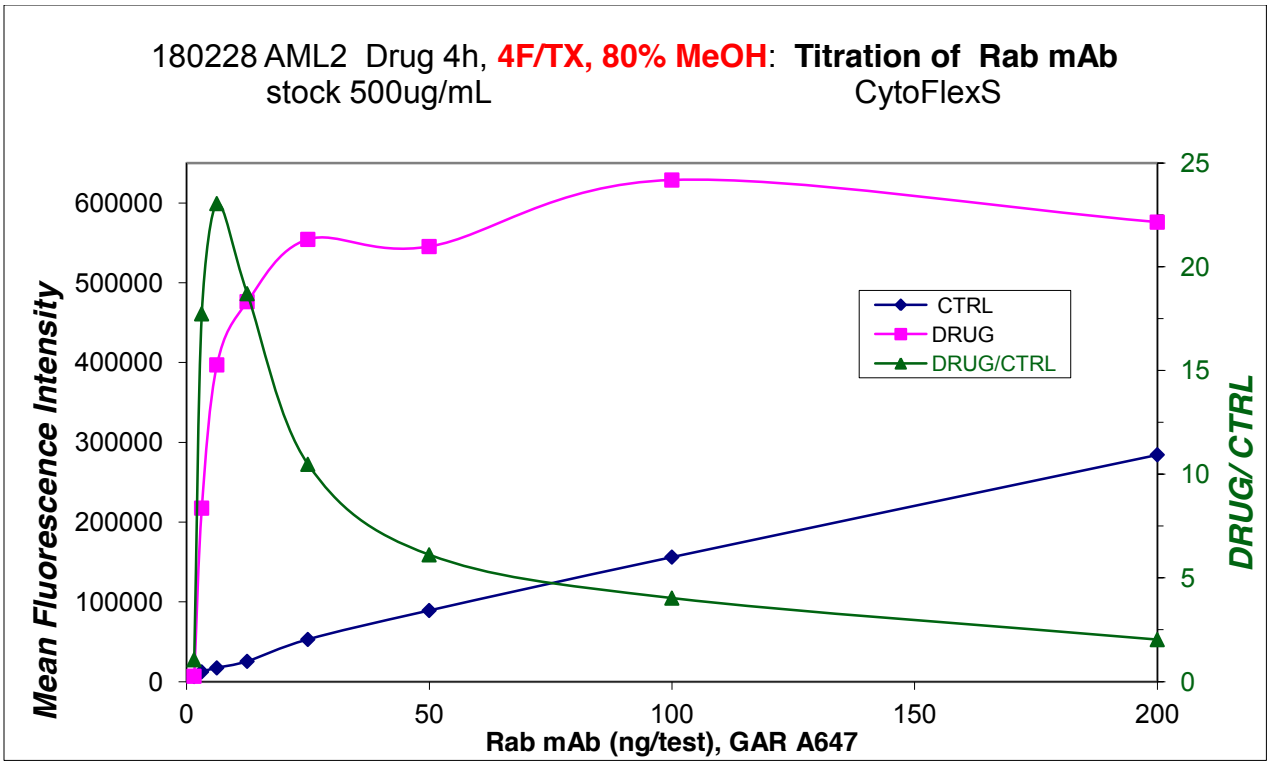


Results: 80% methanol treated cells are not good for the Biolegend pH3 S10 A488 antibody. Since then I have used this antibody to stain other 2% formaldehyde fixed human cell lines.

Antibody Evaluation 2: unconjugated rabbit monoclonal antibody.



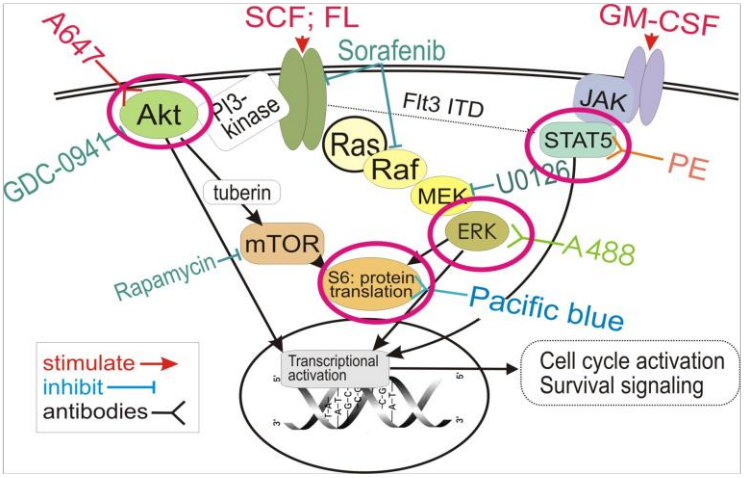




Signal Transduction Analysis by Flow

- Based on combinations of phosphospecific antibodies to monitor transient alterations in specific phosphorylation states
- For clinical samples, applicable to leukemic and normal blood cells
- Leukemia patients
  - identify aberrant patterns for prognosis or treatment selection
- Can also be used to monitor effects of targeted agents during treatment (pharmacodynamics)

A four colour "signaling backbone" to study the major signalling aberrations seen in AML patients



Using rapid whole blood processing protocol, Backbone detects constitutive signaling in peripheral blasts of majority of AML patients – typical data analysis

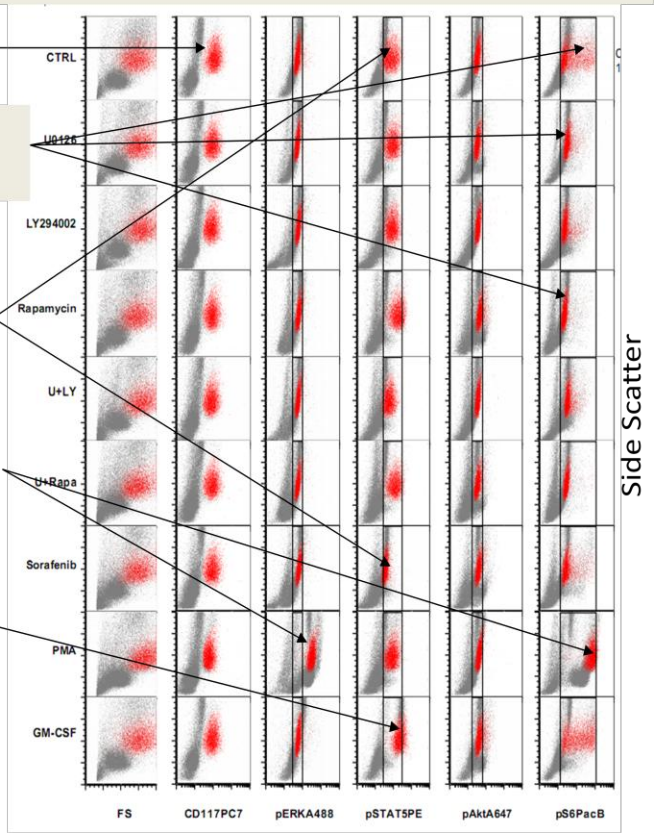
Blasts colour-gated based on CD117

S6 constitutively phosphorylated, responsive to MEK and mTOR inhibition

STAT5 constitutively phosphorylated, responsive to sorafenib

Phorbol ester stimulates ERK and S6 - positive control

GM-CSF stimulates STAT5 in grans - positive control



F/TX Fix and Lyse Method for Whole Blood or Bone Marrow

Date: 23-08-2012

1. Dispense 100µl of PB into 5ml polypropylene BD Falcon tubes.
2. Treat samples if desired with agonists/inhibitors (eg 10 min for PMA; 30 min for inhibitors – do the countdown appropriately allowing 10 sec for addition of reagents to each tube)
3. Fix with 65µl 10% formaldehyde (per 100ul of starting whole blood) for 10 min at RT. (i.e. 4% final concentration formaldehyde). Add formaldehyde to side of tube just above surface so can use same tip for each addition and then vortex hard. Timing is very important (add 10s to each 10 min timing to account for time of manipulation between each tube). .
4. Permeablized with 1ml 0.1165%Triton X-100 for 15 min at 37C. [10% Triton X-100 stock is diluted in PBS (Mg^{++} and Ca^{++} free), and store in dark]. Final concentration is 0.1% TX-100. Timing less critical for this 15 min period.
5. Add 1ml Wash Buffer, mixed & spin down to wash out hemaglobin. Spin down at 2100 rpm in desktop (1000g) for 4 min
6. [[[For storage – if you are not proceeding directly to staining - add 0.5ml Freezing Medium (see below) and keep in –20^{o}C freezer, or proceed to Step 7 directly]]].
7. Some epitopes require alcohol to unmask the epitope. Omit this step if not needed. Add 1ml 80% Methanol/ 0.9% NaCl for 10 min, on ice. Timing not critical but not recommended for longer than overnight because autofluorescence increases with time. Some epitopes eg ERK, Akt, and S6 do not require 80% methanol denaturation but STAT1, 3 and 5 do.
8. Spin down at 2100 rpm in desktop (1000g) for 4 min
9. Loosen pellet and wash cells with 2ml of Wash Buffer. Spin down (2100 rpm in desktop for 4 min).
10. Ready for staining with conjugated antibodies. For indirect labeling, first stain with first antibody for 30 min at RT, wash with 2 ml wash buffer 2100 rpm 4 min, then add second antibody conjugates to all tubes at same time for 30 min at RT, wash again with 2 ml Wash Buffer 2100 rpm for 4 min. (Filter samples if needed as the debris from RBC lysis may clog flow cytometer – add 1 ml to the tube with cells and the second ml to a fresh labeled tube into which you will filter the cells. Home-made filter is 35um nylon mesh taped to end of 1ml pipet tip
11. Read on flow cytometer.

We use Mg^{++} and Ca^{++} free PBS.

Wash Buffer store in fridge**:**
4% FBS in PBS

Freezing Medium store in -20C freezer**:**
10% glycerol
20% FBS
70% RPMI 1640

Please don't use ethanol because cells are unstable in ethanol. **Methanol** is the alcohol to use.
Can 'spike' your whole blood with your control Jurkat cells – add about 10^6 Jurkats for 5-10 million white cells that would be in 100ul of whole blood

This method is also used for cell lines. Concentrate cells to 1 million / 100ul tissue culture medium.

F/TX Fix and Perm Method for cell lines

Date: 14-03-2018

Usually 1/10 amount of stimuli and inhibitors use for whole blood.
Centrifuge speed is lower, [about half (1100rpm) that of blood cells (2100rpm)].

1. Trypsinize cells and spin down in 14ml round bottom polypropylene BD Falcon tubes.
2. Resuspend trypsinized cells to 100µl with Wash Buffer.
3. Fix with 25µl 10% formaldehyde (i.e. 2% final concentration formaldehyde) for 10 min at RT. Add formaldehyde to side of tube just above cell surface so can use same tip for each addition and then vortex hard. Timing is very important (add 10s to each 10 min timing to account for time of manipulation between each tube). .
4. Permeablized with 1ml 0.1%Triton X-100 for 15 min at 37C. [10% Triton X-100 stock is diluted in PBS (Mg^{++} and Ca^{++} free), and store in dark]. Timing less critical for this 15 min period.
5. Add 1ml Wash Buffer, mixed & spin down. Spin down at 1100 rpm in desktop (500g) for 4 min
6. [[[For storage – if you are not proceeding directly to staining - add 0.5ml Freezing Medium (see below) and keep in –20^{o}C freezer, or proceed to Step 7 directly]]].
7. Ready for staining with conjugated antibodies
8. Read on flow cytometer.

***We use only Mg^{++} and Ca^{++} free PBS.

Solutions store RT in dark
10% formaldehyde
0.1% Triton X-100 (10% stock solution) in PBS

Wash Buffer store in fridge**:**
4% FBS in PBS

Freezing Medium store in -20C freezer**:**
10% glycerol
20% FBS
70% RPMI 1640

Maximum 20 million fixed/permed cells per ml freezing medium store in -20C freezer

Antibody staining cells for flow cytometry

1. Pellet cells in 5mL polystyrene flow tube
2. Aspirate all supernatant
3. Add 10uL of antibody cocktail to cell pellet
4. Vortex tube to mix
5. Stain 30 minutes at RT in dark
6. Add 2 mL Wash Buffer
7. Vortex tube
8. Spin down cells
9. Aspirate all supernatant
10. Store tube with pellet in fridge or on ice if not running flow immediately
11. Just before flow run vortex tube to loosen cell pellet
12. Add 350-500uL Run buffer
13. Or for DNA stain, add 350-500uL DAPI stain (1ug/mL WBE) incubate 1hr at RT in dark
14. For long flow runs (more than 2 hours), keep DAPI stained samples on ice and load each tube manually

Buffers

***We use only Mg^{++} and Ca^{++} free PBS.

Wash Buffer store in fridge**:**
4% FBS (fetal bovine serum) in PBS

WBE store in -20C freezer**:**
Wash Buffer with 5mM EDTA
0.5mL EDTA 0.5M stock / 50 mL Wash Buffer

Run Buffer store in fridge:
0.1% formaldehyde/PBS

DAPI staining Buffer make fresh daily, discard leftover :
1ug/mL WBE, incubate 1hr at RT in dark before flow
EDTA prevents cell clumping
(DAPI stock = 1mg/mL water, store in fridge in dark)

Antibody diluent and antibody cocktail store in fridge

Flow Reagents

10% ultra pure formaldehyde EM grade
1L, Poly Sciences 04018, store at RT in dark

Triton X-100 10% aqueous solution
Surfact-Amps X-100
10mL ampule, Thermo Scientific Product # 28314, store at RT in dark

DAPI = 4', 6-diamidino-2-phenylindole.2HCl , FW 350.2
10mg, Sigma D9542
DAPI stock solution = 1mg/mL water, store in fridge in dark

EDTA = ethylenediamine tetraacetic salt solution pH 7.5 - 8.5, 0.5M, FW336.21
100mL, Sigma E7889, store in fridge

Glycerol
Sigma G2025, store at RT

LPS (Lipopolysaccharides) from *Escherichia coli* O127:B8, Sigma L4516
1mg/mL PBS, -20C; working 10ug/mL 4C for six months;
use 1uL per 100uL blood = final concentration 100ng/100uL blood

PMA LC Labs P-1680
40uM Ethanol -20C; 1uL per 100uL blood=final concentration 400nM

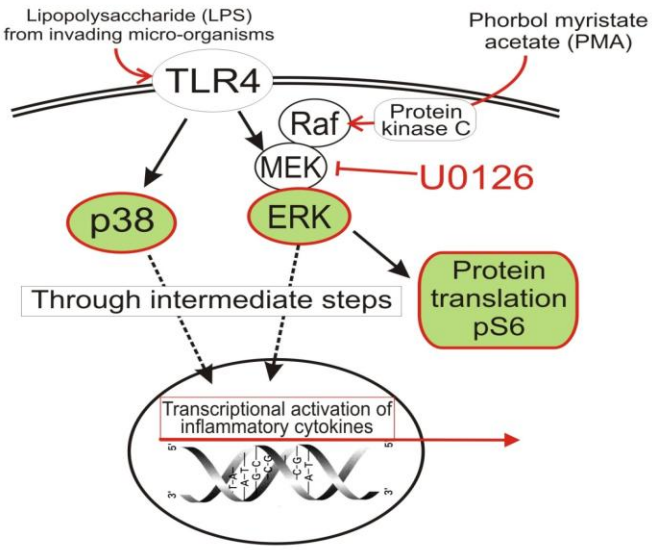
U0126 (Mek inhibitor), LC Labs U-6770
10mM Ethanol -70C, small working aliquot -20C
1uL per 100uL blood=final concentration of 100uM

Dako Antibody Diluent
125mL Cat # S0809, store in fridge

Antibodies store in fridge in dark
CD14-PC7, Beckman Coulter, A22331

CD45-KrO, Beckman Coulter, A96416

CELL SIGNALING LAB



Antibodies
Phosphorylated ERK (A647), p38 (A488) and S6 ribosomal protein (Pacific Blue)
+ surface staining for CD14 and CD45

Tubes
1) Untreated control
2) LPS (activates ERK, p38 and S6 in monocytes only)
3) PMA (activates ERK but not p38 in all leukocytes; lymphocytes most responsive)
4) MEK inhibitor U0126
5) U0126 + LPS (inhibits ERK and S6 activation in monocytes, but not p38)
6) U0126 + PMA (inhibits ERK and S6 activation in all leukocytes)

EXPERIMENT LAYOUT

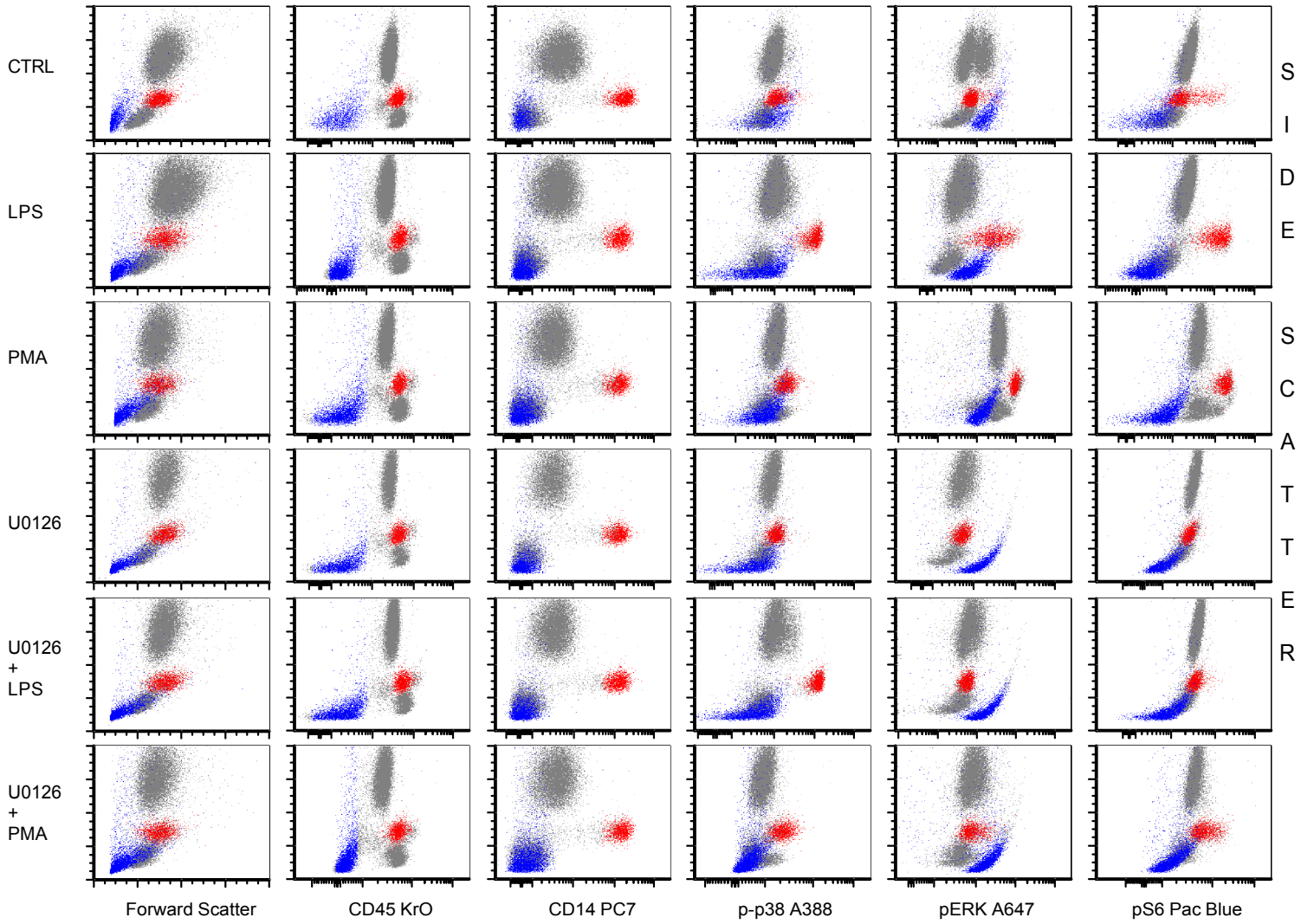
Tubes
1. CTRL
2. LPS 100ng/100uL, 10' 37C
3. PMA 400nM, 10' 37C
4. U0126 100uM, 30' 37C (20', add PMA, 10' = 30' total)
5. U+LPS
6. U+PMA

Procedure
1. Aliquot 100uL blood into 5mL polypropylene tubes (Falcon)
2. Add 1uL of U0126 to tubes 4, 5, and 6; incubate 20' at 37C
3. At 20' take out the U0126 tubes and arrange tubes in the of order of the experiment layout table

	TIME TO ADD		
Tube	Stimulus	Formadehyde	0.1% TX-100
LPS	0:00	10:00	20:00
U+LPS	0:10	10:10	20:10
PMS	0:20	10:20	20:20
U+PMA	0:30	10:30	20:30
U0126	0:40	10:40	20:40
CTRL	0:50	10:50	20:50

171017 PBDH 4FTX, no MeOH: phospho-p38 A488+pERK A647+pS6 Pac Blue+CD45 KrO+CD14 PC7, 30min RT

BD
W180404SC



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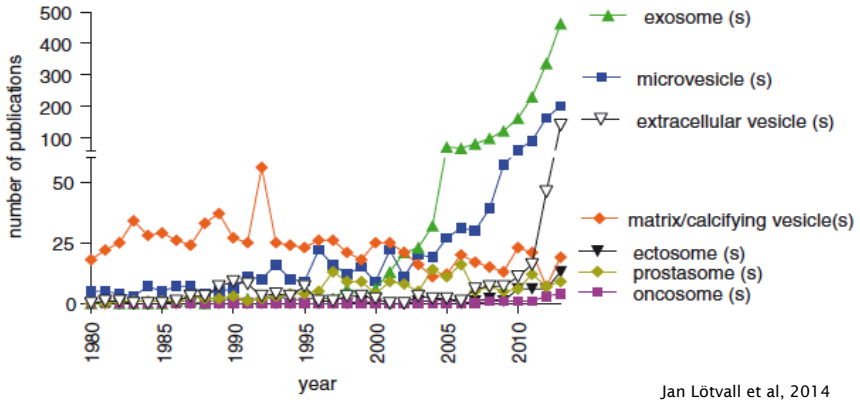
Extracellular Vesicles

Basics - Pitfalls - Artefacts

Andreas Spittler

Core Facility Flow Cytometry & Surgical Research Laboratories

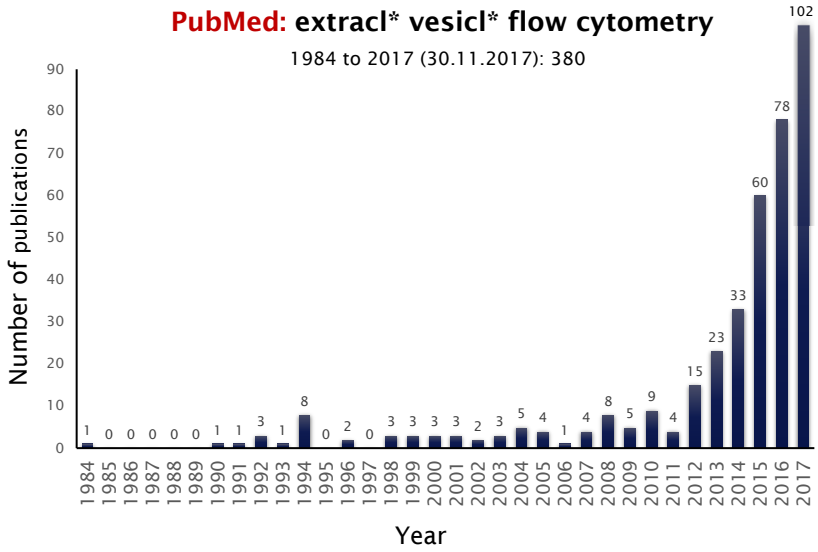


Jan Lötvall et al, 2014

extracell* vesicl* 2016 = 1119 – PubMed, 09.01.2017, 13:58
extracell* vesicl* 2017 = 1339 – PubMed, 22.04.2018, 10:18
extracell* vesicl* 2018 = 627 – PubMed, 22.04.2018, 10:20
extracell* vesicl* 2018 = > 2000

PubMed: extracl* vesicl* flow cytometry

1984 to 2017 (30.11.2017): 380



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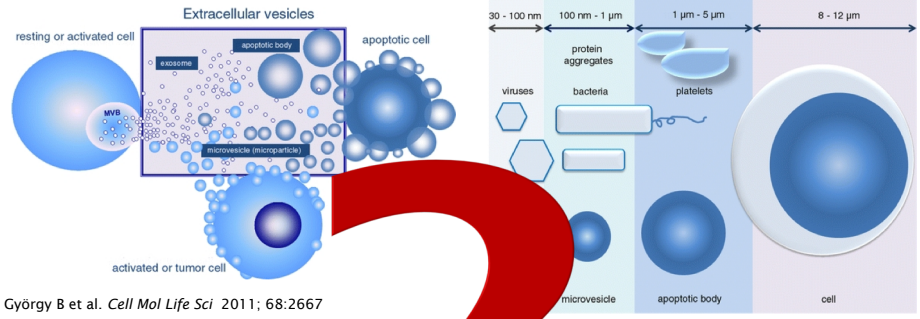

György B et al. *Cell Mol Life Sci* 2011; 68:2667

	Exosomes	Microparticles	Apoptotic bodies
Size	50–100 nm	100–1000 nm	1000–4000 nm (Théry et al., 2009; Mause and Weber, 2010; György et al., 2011)
Biogenesis	Exocytosis from MVB/internal compartments	Cell surface ecto	Blebbing and fragmentation (Théry et al., 2009; Mause and Weber, 2010; György et al., 2011)
Markers	CD63, CD81, CD9, LAMP1 high MHC-I/II (Denzer et al., 2000)	Tissue factor (De Rop et al., 2011; Zwicker et al., 2011), cell surface markers of cell origin, PS	PS, genomic DNA, histones (Mause and Weber, 2010)
Functions	Pro- and anti-inflammatory, antigen presentation (Aline et al., 2004; Kim et al., 2007; Schorey and Bhatnagar, 2008; Ramachandra et al., 2010)	Pro- and anti-infl tigen presentation (Distler et al., 20 t al., 2007; Couper et al., 2010; Ram l., 2010; Sadallah et al., 2011b), coagulation (Owens and Mackman, 2011; Zwicker et al., 2011)	Pro- and anti-inflammatory (Savill et al., 2002), antigen presentation (Climent et al., 2011)

Adapted from. Zheng Lung Ling et al. Frontiers in Immunology 2011; 29:2

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Extracellular Vesicles

Lötvall J, Hill AF, Hochberg F, Buzás EI, Di Vizio D, Gardiner C, Gho YS, Kurochkin IV, Mathivanan S, Quesenberry P, Sahoo S, Tahara H, Wauben MH, Witwer KW, Théry C

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles.

J Extracell Vesicles. 2014, 3:26913

van der Pol E, Böing AN, Gool EL, Nieuwland RJ

Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles.

Thromb Haemost. 2016, 14:48-56

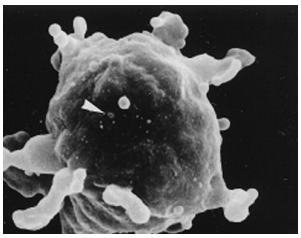
Brisson AR, Tan S, Linares R, Gounou C, Arraud N

Extracellular vesicles from activated platelets:
a semiquantitative cryo-electron microscopy and immuno-gold labeling study.

Platelets. 2017 Jan 19:1-9

Where do we find microparticels?

- Basal levels of MPs in healthy individuals detectable
- Indicate cell proliferation, stimulation and cellular destruction



Picture: McVey M et al. AJP – Lung Cellular and Molecular Physiology 2012; 303:L364

- blood
- plasma
- bone marrow
- cerebro-spinal fluid
- pleural fluid
- joint fluid
- vitreous liquid, tears
- urine
- saliva

Zheng Lung Ling et al. Frontiers in Immunology 2011; 29:2

Barteneva *et al. BMC Cell Biology* 2013, **14**:23
http://www.biomedcentral.com/1471-2121/14/23

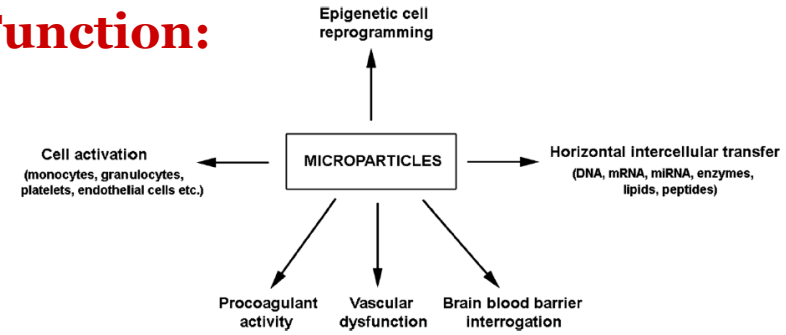
BMC Cell Biology

REVIEW Open Access

Circulating microparticles: square the circle

Natasha S Barteneva[1,2*], Elizaveta Fasler-Kan[3,4], Michael Bernimoulin[5], Joel NH Stern[6], Eugeny D Ponomarev[7,8], Larry Duckett[9] and Ivan A Vorobjev[10]

Function:



Extracellular Vesicles in Probands with Healthy Status

Disease	MPs plasma levels	Reference
Cord blood	Elevated MPs levels or activity comparing with mother's plasma	Uszynski et al., 2011; Schweintzger et al., 2010; 2011
Healthy smokers	Elevated EMPs levels; Diminished MP levels	Gordon et al., 2011; Grant et al., 2011
Healthy donors	MP levels	Berckmans et al., 2001; Bretelle et al., 2003
Normal pregnancy	Elevated MPs levels	Bretelle et al., 2003
Strenuous physical exercise	Elevated PMPs and PMN-MPs	Chaar et al., 2011
Gender	Elevated $CD61^+$ MPs in men; no difference	Caby et al., 2005; Toth et al., 2007; Grant et al., 2011
Climacteric	Lowered PMPs levels, no impact on EMPs levels	Rank et al., 2012
Age (<18 years)	Elevated MPs levels	Proulle et al., 2005
Age (geriartric patients)	Decrease EMPs, altered MPs response to infection	Forest et al., 2010
High-fat meal	Elevated cycling blunts of $CD18^+$ and $CD11a^+$ MMPs and EMPs levels	Strohacker et al., 2012
Obesity	Elevated MPs levels; elevated $CD144^+$EMPs	Goichot et al., 2006; Esposito et al., 2006; Gunduz et al., 2012
Endotoxemia (*E.coli* LPS) in healthy volunteers	Elevated TF^+ MPs	Aras et al., 2004; Woei-A-Jin et al., 2012*

Barteneva *et al. BMC Cell Biology* 2013, **14**:23

Extracellular Vesicles in Various Diseases

Disease	MPs plasma levels	Reference
AUTOIMMUNE DISEASES		
Ankylosing spondylitis	No differences between patient and control groups in EMPs and PMPs levels	Sari et al., 2012
Anti-phospholipid syndrome	Elevated MPs levels; TF^+ EMPs, monocyte-derived MPs	Joseph et al., 2001; Dignat-George et al., 2004; Jy et al., 2007; Vikerfors et al., 2012
Arthritis	Elevated MPs levels	Berckmans et al., 2002; Boilard et al., 2010
Acute inflammatory bowel disease	Elevated MPs levels; elevated TF^+ MPs	Andoh et al., 2005; Palkovits et al., 2012
CARDIOVASCULAR DISEASES		
Acute coronary syndrome	Elevated EMPs levels; Elevated Annexin V^+, EMPs and PMPs levels	Bernal-Mizrahi et al., 2003; Biassuci et al., 2012
Acute pulmonary embolism	PMPs elevated	Bal et al., 2010
Arterial erectile dysfunction	Elevated EMPs levels	La Vignera et al., 2012; Condorelli et al., 2012
INFECTIOUS DISEASES		
Hepatitis C	Elevated T-cell MPs levels correlated with severity of disease	Kornek et al., 2011, 2012
Hepatitis C with cirrhosis	Elevated MPs levels comparing with HepC; elevated MPs from CD4+ and $CD8^+$ T-cells	Brodsky et al., 2008
HIV	Elevated MPs and EMPs levels; upregulation TF and P-selectin	Gris et al., 1996; Holme et al., 1998; Corrales-Medina et al., 2010; da Silva et al., 2011; Mayne et al., 2011

Barteneva *et al. BMC Cell Biology* 2013, **14**:23

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Extracellular Vesicles in Cancer Patients

Disease	MPs plasma levels	Reference
Acute myeloid leukemia	Elevated MPs levels; decreased during chemotherapy and increased during remission;elevated $CXCR4^+$- MPs; elevated PMPs and myeloblast-derived MPs	Kalinkovich et al., 2006; Szczepanski et al., 2011; Van Aalderen et al., 2011
Acute lymphoid leukemia	MPs in bone marrow aspirate	Savasan et al., 2004
Acute promyelocytic leukemia	Elevated $CD33^+TF^+$ MPs	Ma et al., 2013
B-cell chronic lymphoid leukemia	Elevated MPs levels	Ghosh et al., 2009
Gastric cancer	Elevated MPs and PMPs levels	Kim et al., 2003; Baran et al., 2010
Glioblastoma multiforme	Elevated procoagulant MPs	Sartori et al., 2011
Gynecological cancer	MPs levels are not elevated	Zahra et al., 2011
Hepatocellular carcinoma	Elevated MPs levels	Brodsky et al., 2008
Lung cancer	Elevated MPs levels	Kanazawa et al., 2003
Non-small cell lung cancer	Elevated $AnnexinV^+$-MPs	Fleitas et al., 2012
Pancreas cancer	Elevated TF^+ MPs	Thaler et al., 2012
Prostate cancer	Elevated TF^+ MPs; elevated MPs levels	Haubold et al., 2009; Coumans et al., 2010
Different tumor types	Elevated procoagulant MPs levels	Manly et al., 2010; Thaler et al., 2011
Cancer with thromboembolic complications	Elevated MPs levels	Zwicker JI et al., 2009
Tumor surgery (tumor mass removal)	MPs decreased	Zwicker et al., 2009; Sartori et al., 2011

Barteneva *et al. BMC Cell Biology* 2013, **14**:23

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Journal of Extracellular Vesicles

2 July 2015

ORIGINAL RESEARCH ARTICLE

Physical exercise induces rapid release of small extracellular vesicles into the circulation

Carsten Frühbeis[1], Susanne Helmig[2], Suzan Tug[2], Perikles Simon[2]* and Eva-Maria Krämer-Albers[1]*

[1]Molecular Cell Biology, Johannes Gutenberg-University Mainz, Mainz, Germany; [2]Department of Sports Medicine, Johannes Gutenberg-University Mainz, Mainz, Germany

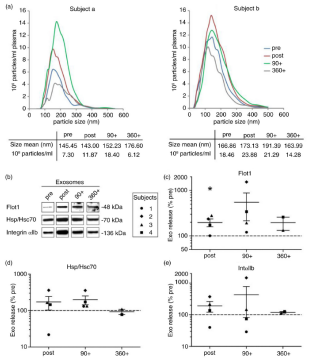

Fig. 3. Effect of running exercise on plasma small EVs. Plasma samples were taken pre, post, 90 post (90+) and 360 post (360+) treadmill exercise. Pellets of 100,000 × *g* were analyzed for particle size and concentration by NTA [(a) subjects a and b] and for protein composition by Western blotting [(b) subjects 1–4]. Signal intensities of Flot1 (c), Hsp/Hsc70 (d) and Itnt1db (e) were quantified (pre/post/90+: n = 4, 360+: n = 2, *p < 0.05, Student's t-test).

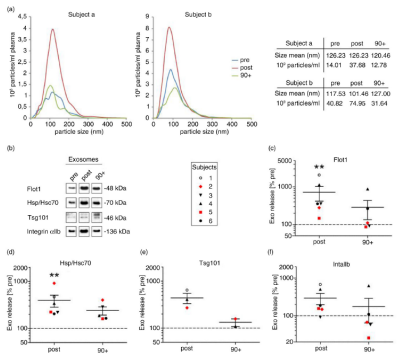

Fig. 1. Effect of cycling exercise on plasma small EVs. Plasma samples were collected pre, post and 90 min post (90+) ergometer exercise, and 100,000 × *g* pellets were prepared. Particle size and concentration were measured by NTA [(a) subjects a and b, age above 30] and protein content was analyzed by Western blotting [(b) subjects 1–6]. Western blot signals of Flot1 [(c) pre/post: n = 6, 90+: n = 5], Hsp/Hsc70 [(d) pre/post: n = 6, 90+: n = 5], Tsg101 [(e) pre/post: n = 3, 90+: n = 2; not detectable in all experiments] and Intα1b [(f) pre/post: n = 6, 90+: n = 5] were quantified (**p < 0.01, Wilcoxon–Kruskal–Wallis test). Subjects older than 30 years are marked in red (subject 2, 38 years; subject 5, 58 years).

"… Taken together, our study revealed that exercise triggers a rapid release of EVs with the characteristic size of exosomes into the circulation, initiated in the aerobic phase of exercise. We hypothesize that EVs released during physical activity may participate in cell communication during exercise-mediated adaptation processes that involve signaling across tissues and organs."

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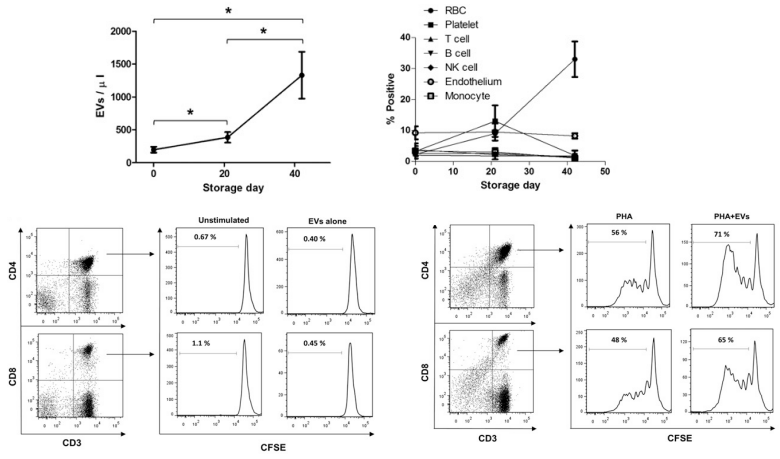
IMMUNOBIOLOGY

BLOOD, 30 JANUARY 2014 • VOLUME 123, NUMBER 5

Exosomes from red blood cell units bind to monocytes and induce proinflammatory cytokines, boosting T-cell responses in vitro

Ali Danesh,[1,2] Heather C. Inglis,[1] Rachael P. Jackman,[1] Shiquan Wu,[1] Xutao Deng,[1] Marcus O. Muench,[1,2] John W. Heitman,[1] and Philip J. Norris[1-3]



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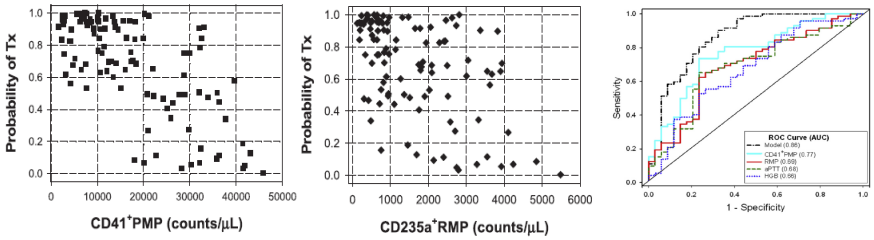
The Journal of Thoracic and Cardiovascular Surgery • Volume 149, Number 1 305
2015

Presurgical levels of circulating cell-derived microparticles discriminate between patients with and without transfusion in coronary artery bypass graft surgery

Wenche Jy, PhD,[a] Orlando Gómez-Marín, MSc, PhD,[b] Tomas A. Salerno, MD,[c] Anthony L. Panos, MD,[c] Donald Williams, MD,[c] Lawrence L. Horstman, BS,[a] and Yeon S. Ahn, MD[a]



"… This study highlights the **importance of microparticles in surgical hemostasis** and supports the conclusion that CABG patients with **low presurgical microparticle levels are at higher risk for transfusion**. …. new strategies to modify levels of microparticles and thereby reduce the risk of excessive surgical bleeding. This, in turn, will translate into substantial reduction in the number of transfusions required and, ultimately, into overall improvement of transfusion practice. …"

Exosome levels in human body fluids: A tumor marker by themselves?

Capello F. et al. *European Journal of Pharmaceutical Sciences* 2017, 96:93–98

Cancer type	Marker(s)	Body fluid	Method	Ref.
Hematological tumors	CD9, CD13, CD19, CD30, CD38, CD63	Serum	FACS	Caivano et al., 2015
Prostate cancer	CD9, CD63	Urine	TR-FIA	Duijvesz et al., 2015
Prostate cancer	Survivin	Plasma	ELISA	Khan et al., 2012
Prostate cancer	–	Plasma	NTA	Nawaz et al., 2014
Prostate cancer	N-linked glycans	Prostatic secretions	MALDI-TOF, HPLC, MS	Nyalwidhe et al., 2013
Colon cancer	Hsp60	Plasma	Western blot	Campanella et al., 2015
Colon-rectal cancer	–	Plasma	FACS	Silva et al., 2012
Melanoma	CD63, caveolin-1	Plasma	ELISA	Logozzi et al., 2009
Melanoma	MIA, S100B	Serum	Electro-chemiluminescence	Alegre et al., 2016
Melanoma	TYRP-2, VLA-4, HSP70, HSP90	Plasma	NTA	Peinado et al., 2012
Pancreatic cancer	Glypican 1	Serum	NTA	Melo et al., 2015
Brest cancer	miR34a, dicer	Plasma	RT-PCR, western blot	Lowry et al., 2015
Gastrointestinal stromal tumor	–	Plasma	FACS	Ogorevc et al., 2013

Why should we measure EVs?

- Correlation with disease activity
- Prognostic value to identify patients with thrombotic or vascular risk
- Information about non accessible tissues (tumors, endothelium, placenta, …)
- Treatment monitoring
- Quality control (blood product)

Methods of Microparticles Detection

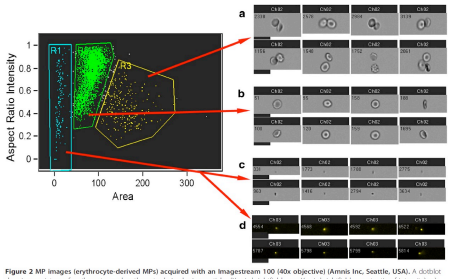
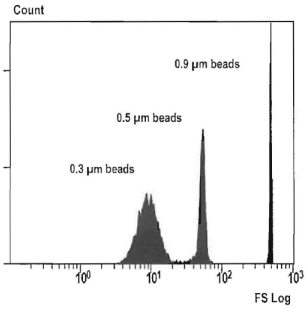
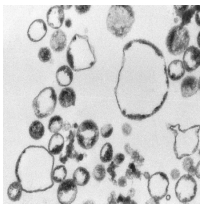
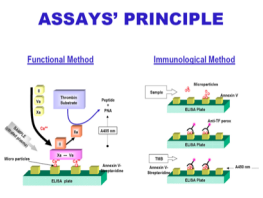
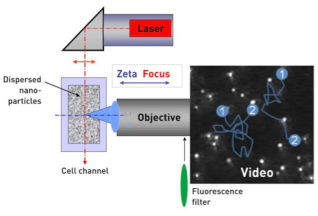


Figure 2 MP images (erythrocyte-derived MPs) acquired with an Imagestream 100 (40x objective) (Amnis Inc, Seattle, USA). A dotblot showing a mixture of erythrocytes and erythrocyte-derived microparticles (X-axis-brightfield area, Y-axis-brightfield aspect ratio of intensity). **a.)** Multiple erythrocytes (region R3 on dotblot); **b.)** Single erythrocytes (region R2 on dotblot); **c.)** Microparticles (brightfield) (region R1 on dotblot); **d.)** Microparticles stained with calcein AM (images from calcein AM-channel are particles taken from R1-region).









Methods of Microparticles Detection

Method	Quantification	Cell origin and/or function identification	MPs size distribution	Limitations	References
Electron microscopy	Limited	Limited (only for single labeling by immunoelectron microscopy)	Yes, but might be subjective due to limited number of measurements	Artifacts due to specimen preparation for negative contrast (drying, application of contrasting solution etc.)	Hess et al., 1999; Distler et al., 2005; Lima et al., 2009; Witek et al., 2009; Porro et al., 2010; Duarte et al., 2012; Gercel-Taylor et al., 2012
Functional assays (procoagulant activity, thrombin generation tests, ELISA-based tests etc.)	Yes (bulk)	No	No	Only information on procoagulant or thrombin generating activity available	Leroyer et al., 2007; Tesselaar et al., 2007; Salzer et al., 2008; Manly et al., 2009; Van der Heyde et al., 2011
Atomic Force Microscopy	Limited	Limited (requires development of AB-coated surfaces)	Yes, but might be subjective due to limited number of measurements	Artifacts due to abundance of cell debri and plasma protein	Salzer et al., 2008; Yuana et al., 2010; Leong et al., 2011; Nantakomol et al., 2012
Light scattering techniques (nanoparticle tracking analysis, submicron particle analysis, dynamic light scattering)	Yes	No*	Yes	Artifacts due to abundance of cell debri and plasma protein – samples requires special purification	Lawrie et al., 2009; Xu et al., 2010; Gercel-Taylor et al., 2012
Western blotting	Semi-quantitative	Yes	No	Requires significant amount of starting material (> 10 μg of vesicular material)	Abid Hussein et al., 2005; Salzer et al., 2008; Sander et al., 2008; Bebawy et al., 2009; Bernimoulin et al., 2009; Gercel-Taylor et al., 2012
Mass-spectrometry	No	Yes, allows identification of multiple proteins	No	Requires significant amount of starting material	Sander et al., 2008; Mayr et al., 2009; Rood et al., 2010
Flow Cytometry	Yes	Yes, allows identification of multiple antigens	Limited	Limited; >300 nm particle range (conventional flow cytometry), presence of protein aggregates may lead to artifacts sensitivity depends on cytometer	Orozco, Lewis, 2010; Zwicker et al., 2010; Ayers et al., 2011; Yuana et al., 2011; van der Heyde et al., 2011
Flow imaging cytometry	Yes	Yes, allows quantification of multiple antigens	No	Limited for bright fluorescence MPs	Van der Heyde et al., 2011

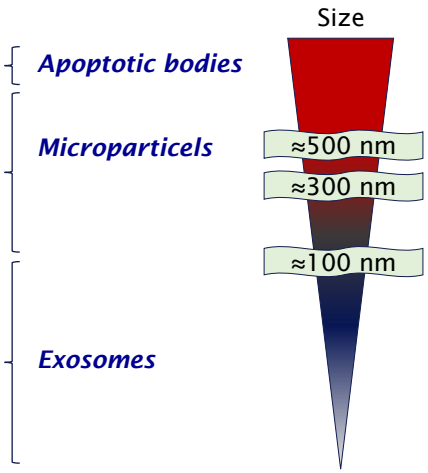
Barteneva et al. BMC Cell Biology 2013, 14:23

MPs Surface Antigen Expression

	Surface antigens	Effect
Red blood cells	**CD235a**	Endothelial binding
Platelets	CD31, **CD41**, CD41a, CD42a, CD42b, CD61, CD62P	Enhanced platelet aggregation Cell-cell interaction of monocytes to endothelial cells
Endothelial cells	CD31, CD34, CD54, CD62E, CD51, CD105, CD106, CD144, CD146	Neutrophil activation Chemotactic attraction of leukocytes
Myelomonocytic cells	**CD15**	Activation of endothelial cells Release of cytokines (IL-6, IL8)
Lymphocytes	CD4, CD8, CD20	Upregulation of NOS2 and COX-2
Monocytes	**CD14**	Activates platelets

Reid VL et al. BJA 2012; 109:503

Flow Cytometry and EVs



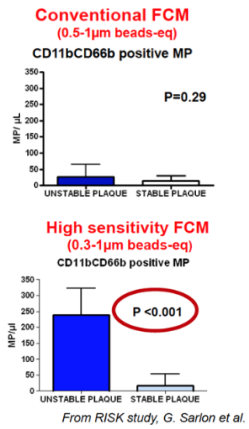
MPs as monitoring parameter?

Plasmatic Level of Leukocyte-Derived Microparticles Is Associated With Unstable Plaque in Asymptomatic Patients With High-Grade Carotid Stenosis

Sarlon-Bartoli G et al. *J Am Coll Cardiol* 2013, 62:1436

	Whole Population		
	Stable Plaque (n = 14)	Unstable Plaque (n = 28)	p Value
Age, yrs	77.0 (62–80)	72.5 (66–76)	0.42
Male	10 (71.4)	22 (78.6)	0.71
Body mass index, kg/m^2	25.4 (24.2–28.1)	25.2 (24.0–27.0)	0.94
Hypertension	12 (85.7)	17 (60.7)	**0.16**
Diabetes mellitus	5 (35.7)	4 (14.3)	**0.13**
Hypercholesterolemia	13 (92.9)	16 (56.5)	**0.03**
Smoker	4 (28.6)	9 (57.1)	0.99
Antiplatelets	14 (100)	26 (92.9)	0.55
Statins	9 (64.3)	15 (53.6)	0.51
ACE inhibitors	7 (50.0)	9 (32.1)	0.32
Neurologic event	1 (7.1)	18 (64.3)	**0.001**
LDL cholesterol, g/l	0.93 (0.8–1.0)	1.2 (0.9–1.2)	**0.15**
hs-CRP, mg/l	3.0 (1.6–4.8)	7.0 (2.0–21.3)	**0.16**
Leukocyte count	8.0 (6.7–9.9)	7.9 (6.6–9.7)	0.58
CD11b66b count	16 (0–234)	240 (147–394)	**0.001**
CD15 count	55 (36–157)	147 (60–635)	**0.009**

Including the small MP subset provides new clinically-related information



From RISK study, G. Sarlon et al.

… EVs are a good tool - but …

Journal of Extracellular Vesicles

COACTION

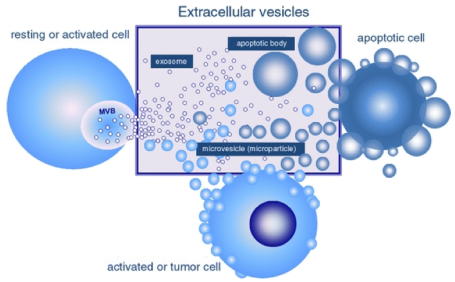
Lötvall J. et al. *J Extracell Vesicles* 2014, 22:26913

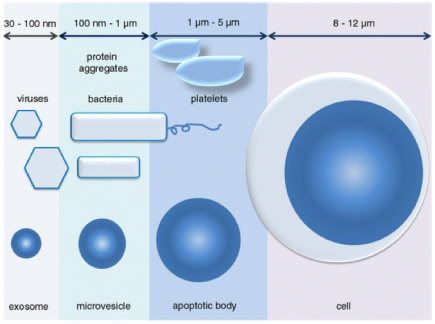
EDITORIAL

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles

Since there is currently **no consensus on a "goldstandard" method** to **isolate and/or purify** EVs, it cannot be claimed that there is an ''optimal'' method that should be uniformly used.
The reader should be aware that the methods that are most efficient probably depend on (a) the specific scientific question asked and (b) on the downstream applications used.

… no consensus on detection, analysis, quantification …





György B et al. *Cell Mol Life Sci* 2011; 68:2667

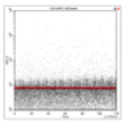
	Exosomes	Microparticles	Apoptotic bodies
Size	50–100 nm	100–1000 nm	1000–4000 nm (Théry et al., 2009; Mause and Weber, 2010; György et al., 2011)
Biogenesis	Exocytosis from MVB/internal compartments	Cell surface ectocytosis	Blebbing and fragmentation (Théry et al., 2009; Mause and Weber, 2010; György et al., 2011)
Markers	CD63, CD81, CD9, LAMP1 high MHC-I/II (Denzer et al., 2000)	Tissue factor (De Rop et al., 2011; Zwicker et al., 2011), cell surface markers of cell origin, PS	PS, genomic DNA, histones (Mause and Weber, 2010)
Functions	Pro- and anti-inflammatory, antigen presentation (Aline et al., 2004; Kim et al., 2007; Schorey and Bhatnagar, 2008; Ramachandra et al., 2010)	Pro- and anti-inflammatory, antigen presentation (Distler et al., 2005a; Huber et al., 2007; Couper et al., 2010; Ramachandra et al., 2010; Sadallah et al., 2011b), coagulation (Owens and Mackman, 2011; Zwicker et al., 2011)	Pro- and anti-inflammatory (Savill et al., 2002), antigen presentation (Climent et al., 2011)

Zheng Lung Ling et al. Frontiers in Immunology 2011; 29:2

10 µm cell

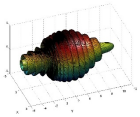
100 nm

… Limitations and Pitfalls

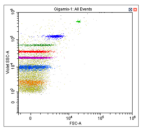


Preanalytics

Biological



Physical - Technical



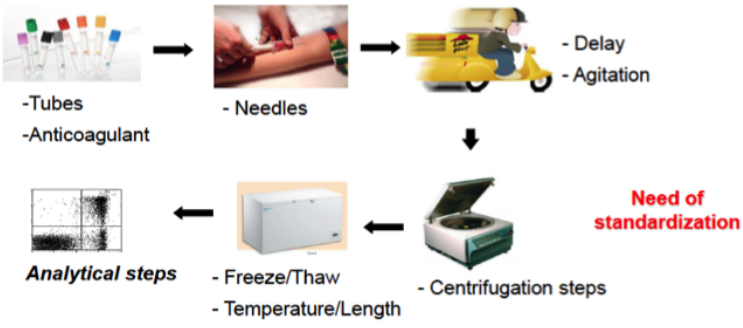
MPs: not Shaken, not Stirred!

Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol

R. LACROIX,*† C. JUDICONE,‡ P. PONCELET,‡ S. ROBERT,* L. ARNAUD,† J. SAMPOL* and F. DIGNAT-GEORGE*†

*UMR1076 INSERM-Aix-Marseille Université, UFR de Pharmacie, Marseille; †Laboratoire d'hématologie, CHU Conception, AP-HM, Marseille; and ‡Biocytex, 140, Chemin de l'Armée d'Afrique, Marseille, France

J Thromb Haemost. 2012, 3:437-46



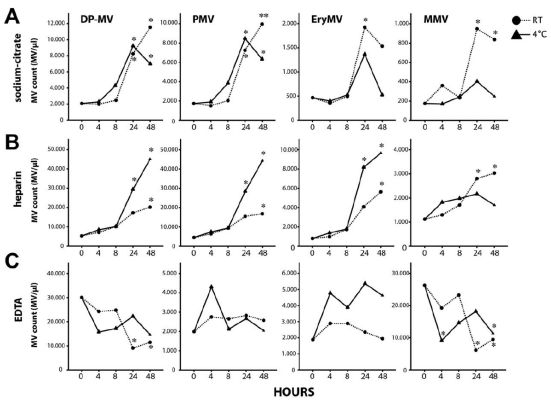
Cytometry PART A

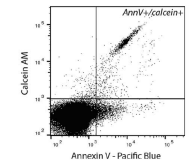
ISAC International Society for Advancement of Cytometry

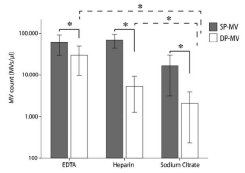
Peripheral Blood Microvesicles Secretion Is Influenced by Storage Time, Temperature, and Anticoagulants

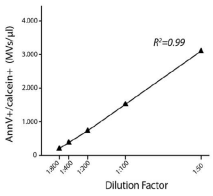
Lukas Wisgrill,[1] Christian Lamm,[2] Julia Hartmann,[2] Falk Preißing,[2] Klaus Dragosits,[2] Annica Bee,[2] Lena Hell,[3] Johannes Thaler,[3] Cihan Ay,[3] Ingrid Pabinger,[3] Angelika Berger,[1] Andreas Spittler[2,4]*

Cytometry Part A • 89A: 663–672, 2016









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Extracellular Vesicles and Flow Cytometry

- Requirements for the measurement of EVs
  - Measurement of the smallest signals which are over the threshold
  - Optimal setting of the threshold
    - Set the FSC or SSC trigger signal so that noise is visible
      - Maximum EPS
    - Decrease the FSC or SSC trigger signal to a channel where only a minimum signal of noise is present
      - ~ 200 EPS
    - … **or use the fluorescence signal as trigger ?**
  - Sheath fluid
    - Non fluorescence
    - Azide free electrolyte solution

… use beads for standardisation (?)

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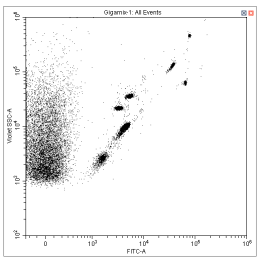
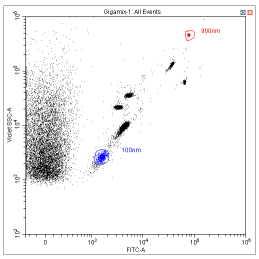
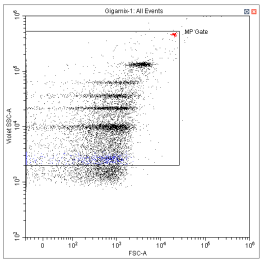
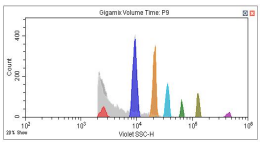
Standardization using Beads

„GigaMix“









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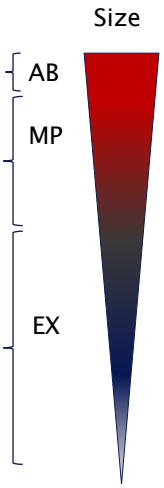
What do we detect?



Harrison et al., JTH 2012, 10:916

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Small Particles

Coincidence

Known problem: platelet-leukocyte complexes

fluorescent platelets / non-fluorescent red cell events

other aggregates / clumping

Harrison et al., JTH 2012, 10:916

Swarm …

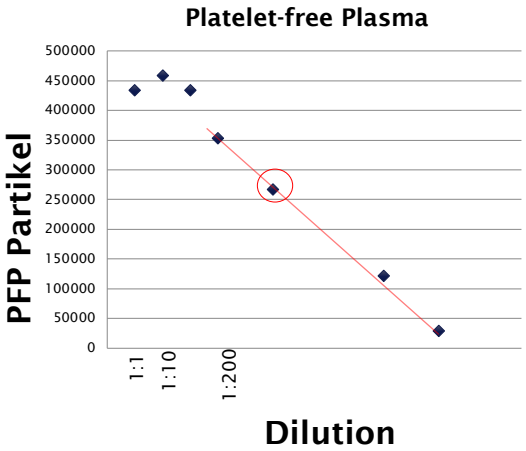
… of small vesicles is counted as larger ***single cell events***

… propability of having ***n*** events simultaneously in the sample stream / passing the laser beam

… underestimates the true concentration of MVs

Van der Pol et al., JTH 2012, 10:919

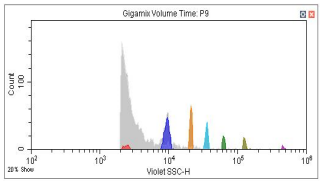
Dilute Your Samples



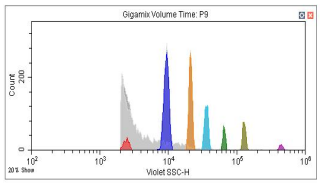
Sample was measured for 1 minute and total particles were counted

Noise – Megamix beads – Flow rate

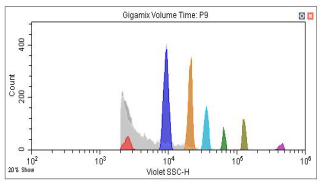
10µl/min

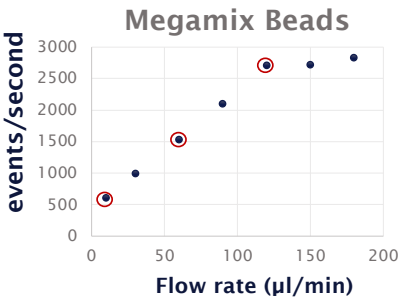


60µl/min



120µl/min





Megamix beads were diluted in destilled water 1:5 and measured with different flow rates

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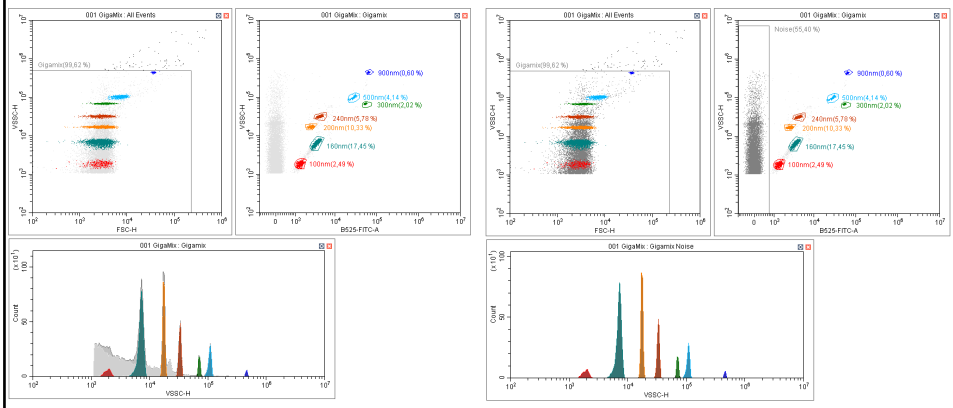
Noise



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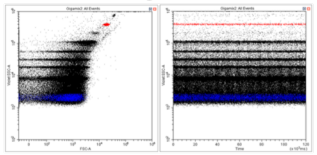
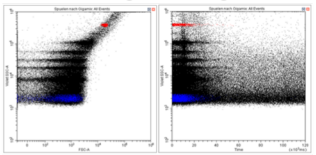
Solutions, Proteins and Background

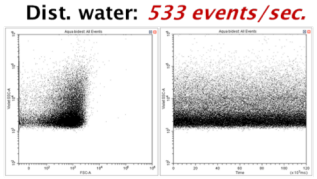
Gigamix



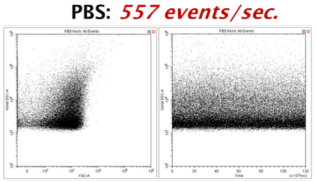
Washing after Gigamix



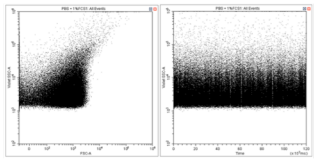
Dist. water: *533 events/sec.*



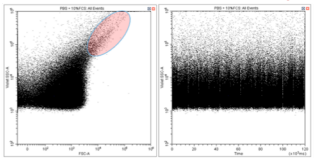
PBS: *557 events/sec.*



PBS + 1% FCS: *2194 events/sec.*



PBS + 10% FCS: *4082 events/sec.*

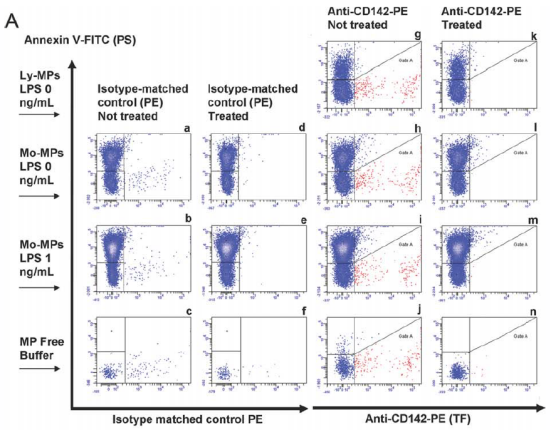


Cytometry PART A

Cytometry Part A • 79A: 990–999, 2011

Fluorescent Particles in the Antibody Solution Result in False TF- and CD14-Positive Microparticles in Flow Cytometric Analysis

Hans Christian D. Aass,* Reidun Øvstebø, Anne-Marie S. Trøseid, Peter Kierulf, Jens Petter Berg, Carola Elisabeth Henriksson



Conclusion

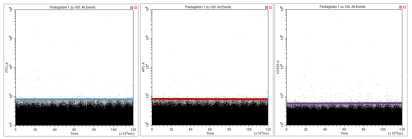
… fluorescent particles, possibly **in the form of antibody aggregates**, are present in commercial antibody solutions and these fluorescent particles can contribute to false TF- and CD14-positive MPs …

… We suggest that inappropriate removal of fluorescent particles in the antibody solutions may also result in discrepancy between flow cytometric detection of TF expression on MPs and lack of TF activity.

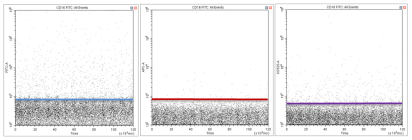
… we propose eliminating them by **centrifugation for at least 5 min at 17,000g, and use the supernatant for MP staining**. This supernatant is free of fluorescent interference and facilitates detection of true MP marker events.

Antibody and Reagent Background

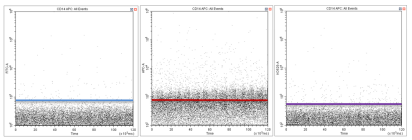
Pentaglobin 1:100 in dist. water



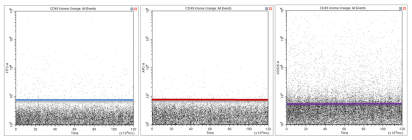
CD16 FITC



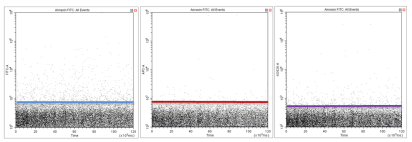
CD14 APC



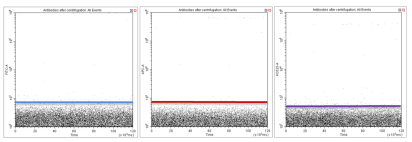
CD45 Krome Orange



Annexin V FITC



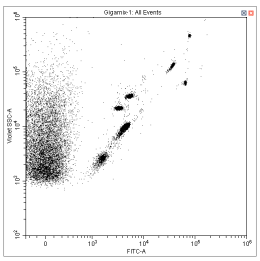
Antibody mix after centrifugation

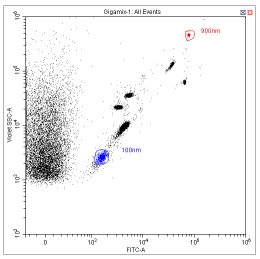


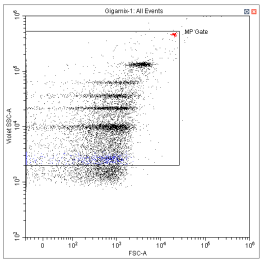
Life was so easy

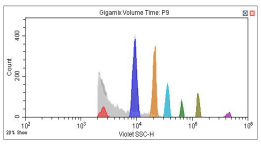
Standardization using Beads

„Megamix“









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Life is much more complicated

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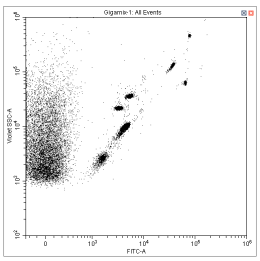
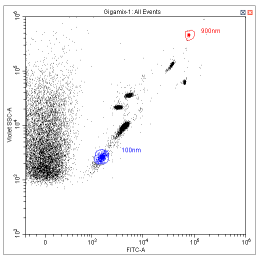
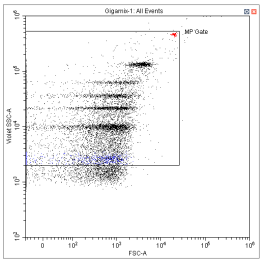
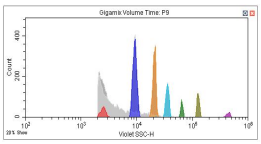
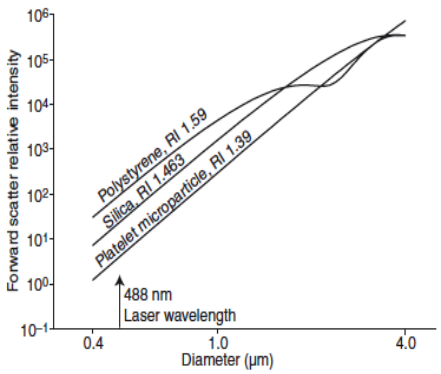
Standardization using Beads

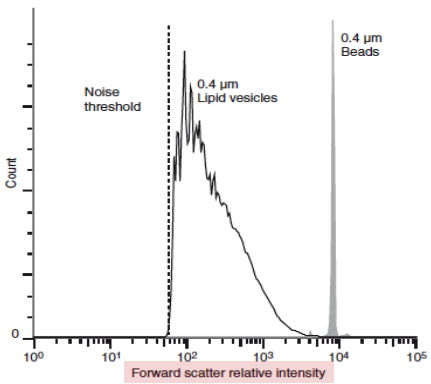
„Megamix“

… or silica beads … or liposomes … or …

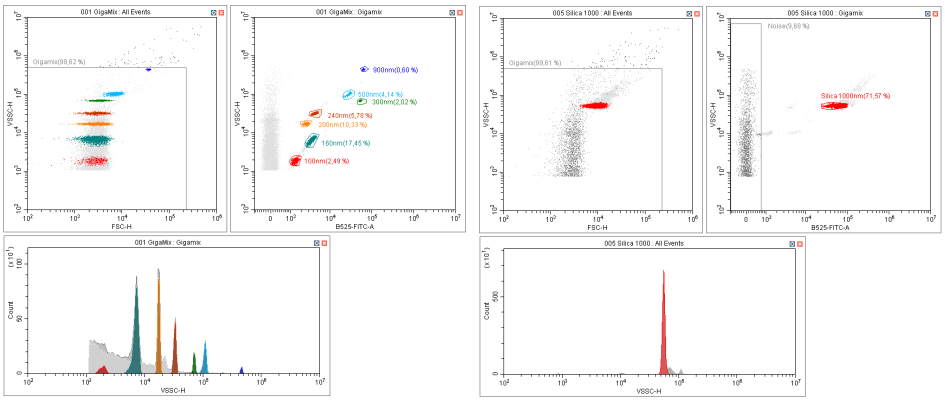
But ..... Refractive Index



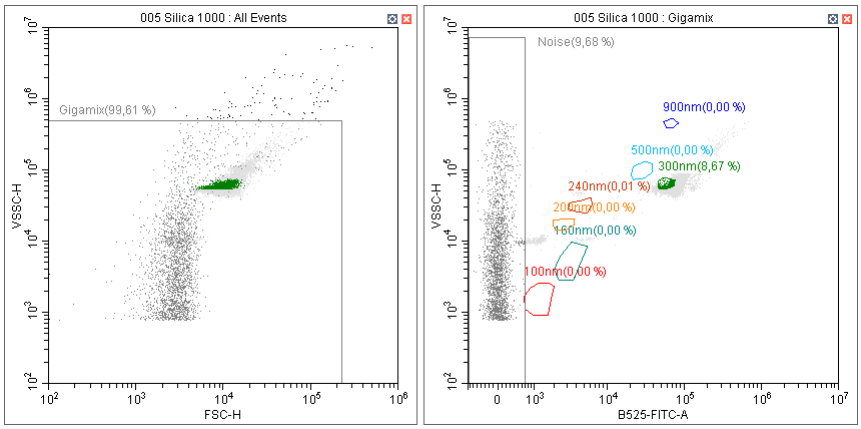


Chandler et al., 2011

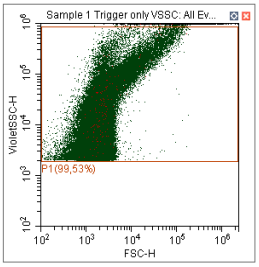
Polystyrene - Silica

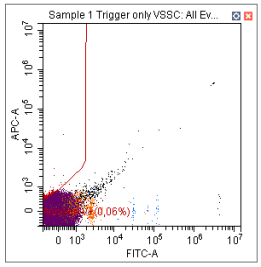


Polystyrene - Silica



Trigger: VSSC vs. Fluorescence Channel

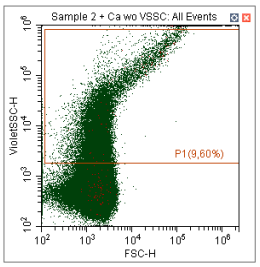


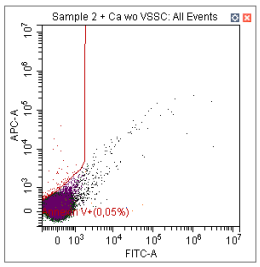


Tube Name: Sample 1
Sample ID:

Trigger only VSSC

Population	Events/µL(V)	Events	% Total	% Parent
All Events	11218,10	258293	100,00%	100,00%
Q1-UR	3,17	73	0,03%	0,03%
Q1-UL	17,11	394	0,15%	0,15%
Q1-LL	11141,66	256533	99,32%	99,78%
Q1-LR	3,82	88	0,03%	0,03%
P1	11165,77	257088	99,53%	99,53%
P2	11167,63	257131	99,55%	99,55%
Annexin ...	6,34	146	0,06%	0,06%



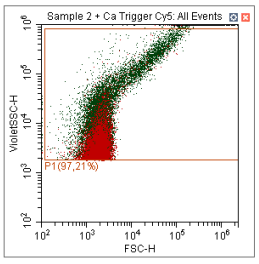


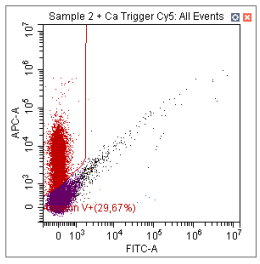
Tube Name: Sample 2
Sample ID:

Trigger Cy5 channel

Population	Events/µL(V)	Events	% Total	% Parent
All Events	14267,08	286055	100,00%	100,00%
Q1-UR	0,05	1	0,00%	0,00%
Q1-UL	0,35	7	0,00%	0,03%
Q1-LL	1368,28	27434	9,59%	99,90%
Q1-LR	0,95	19	0,01%	0,07%
P1	1369,63	27461	9,60%	9,60%
P2	14202,25	284755	99,55%	99,55%
Annexin ...	6,98	140	0,05%	0,05%

Trigger: VSSC vs. Fluorescence Channel

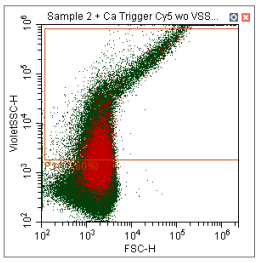


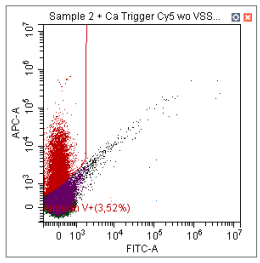


Tube Name: Sample 2
Sample ID:

Trigger only VSSC

Population	Events/µL(V)	Events	% Total	% Parent
All Events	1138,02	22817	100,00%	100,00%
Q1-UR	0,20	4	0,02%	0,02%
Q1-UL	0,60	12	0,05%	0,05%
Q1-LL	1105,20	22159	97,12%	99,91%
Q1-LR	0,25	5	0,02%	0,02%
P1	1106,24	22180	97,21%	97,21%
P2	1115,52	22366	98,02%	98,02%
Annexin ...	337,66	6770	29,67%	29,67%





Tube Name: Sample 2
Sample ID:

Trigger Cy5 channel

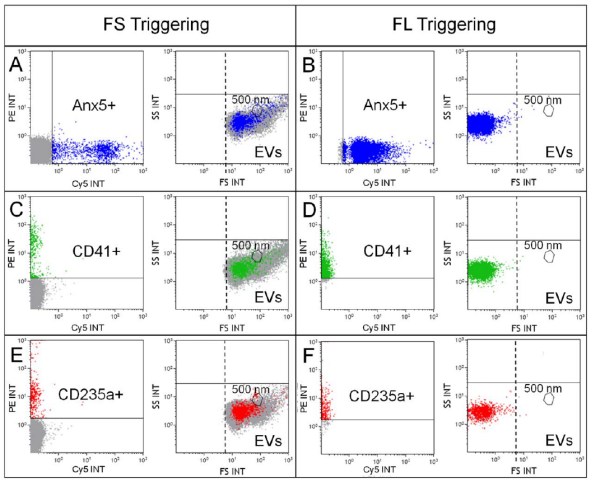
Population	Events/µL(V)	Events	% Total	% Parent
All Events	15738,47	315769	100,00%	100,00%
Q1-UR	0,05	1	0,00%	0,00%
Q1-UL	0,25	5	0,00%	0,01%
Q1-LL	1667,85	33463	10,60%	99,96%
Q1-LR	0,30	6	0,00%	0,02%
P1	1668,45	33475	10,60%	10,60%
P2	15678,76	314571	99,62%	99,62%
Annexin ...	554,04	11116	3,52%	3,52%

Cytometry

Fluorescence Triggering: A General Strategy for Enumerating and Phenotyping Extracellular Vesicles by Flow Cytometry

Nicolas Arraud, Céline Gounou, Delphine Turpin, Alain R. Brisson

Cytometry Part A • 89A: 184–195, 2016



Enhanced Forward Scatter Module+ on the MoFlo™ Astrios*: Small Particle Applications

Dr. Carley D. Ross, Alan Dean Ph.D., Kerry Weinberg, Robin Morris, Blake McCarty, and Nicole Lewis

Beckman Coulter, Inc., Flow Cytometry Business Center, Fort Collins, CO, USA

BECKMAN COULTER
Life Sciences

PURPOSE

Demonstrate the ability of the FSC Module to resolve and sort small (0.2 µm) and large particles (30 µm) simultaneously on two Forward Scatter Parameters.

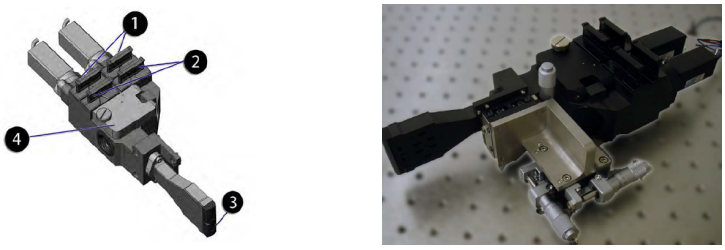


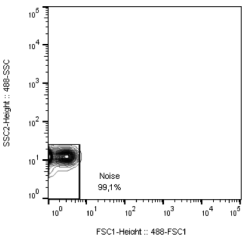
Figure 1. Forward Scatter Module. 1. Neutral density filters in front of the FSC PMTs. 2. Laser blocking filters. 3. Removable FSC cap. 4. Beamsplitting cube.

Sorting !!!

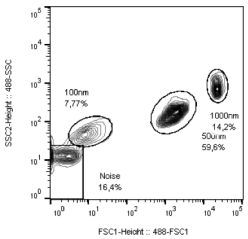
65,000 events/sec

Nozzle tip: 70µm
Pressure diff.: 0.3

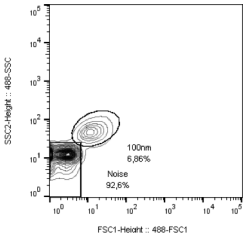
Noise



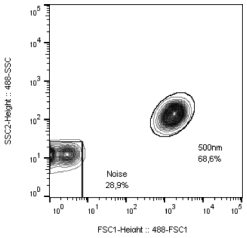
Sort – Silica Beads:
100nm, 500nm, 1,000nm



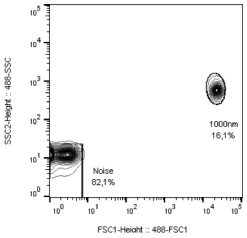
Reanalysis: 100nm



Reanalysis: 500nm



Reanalysis: 1,000nm



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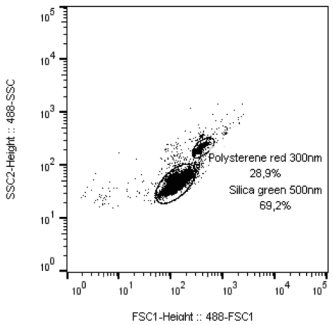
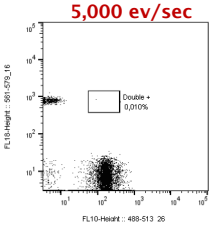
Silica and Polystyrene Beads



5,000 ev/sec



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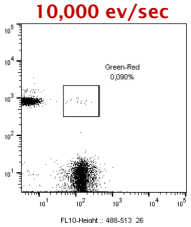

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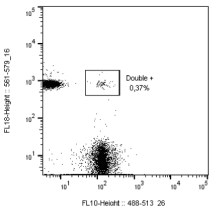

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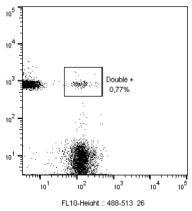
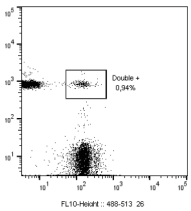
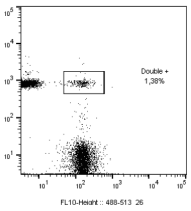


70,000 ev/sec



120,000 ev/sec



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Contents lists available at ScienceDirect

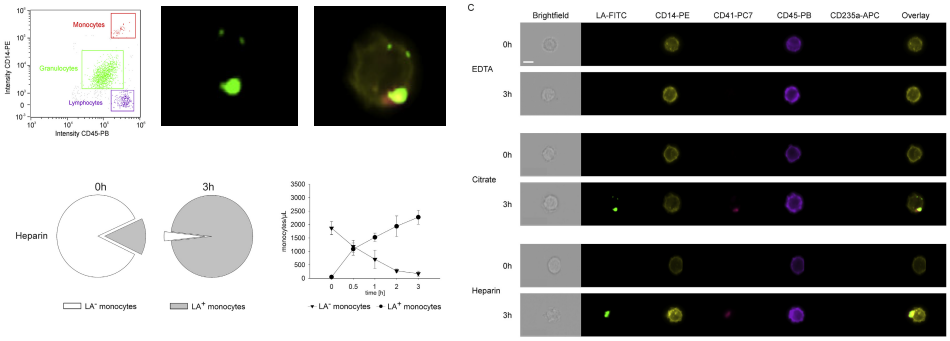
Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of extracellular vesicles in whole blood: Influence of pre-analytical parameters and visualization of vesicle-cell interactions using imaging flow cytometry

Birgit Fendl [a], René Weiss [a], Michael B. Fischer [a], Andreas Spittler [b], Viktoria Weber [a,*]

Biochemical and Biophysical Research Communications 478 (2016) 168–173



Data Analysis

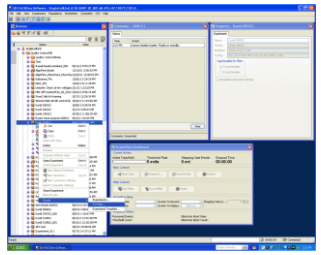
WITH FLOWJO

Cytometry Analysis Software

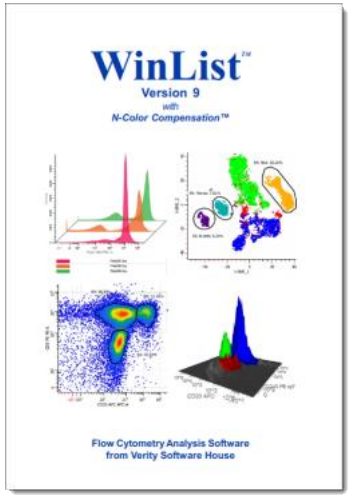
FLOWJO®

Infinicyt™
Flow Cytometry Software

KALUZA



BD
DIVA



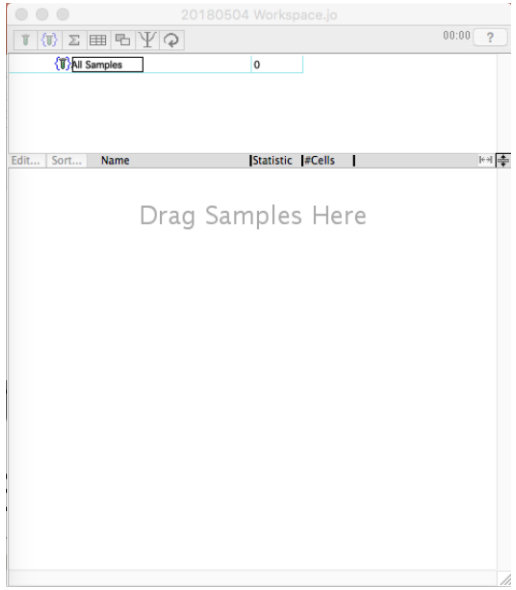
Flowlogic™
Flow Cytometry Analysis Software

De Novo™ Software
Turning Cytometry Data Into Results

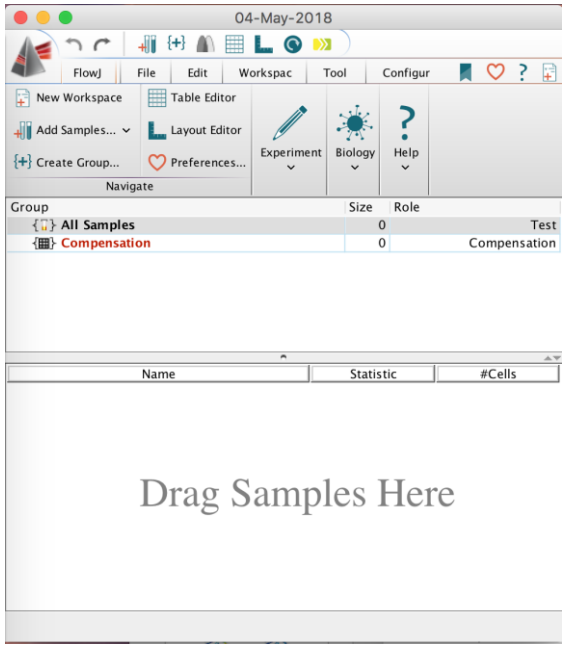
FCS6 Express
FLOW plus IMAGE

2 versions of FlowJo

V 9.9.6. Mac



V 10.4.2. Java ~~"PC"~~



How do you analyze flow-cytomery data?

Identify population of interest
=> gating

Get statistics
=> e.g. MFI

Compare results
=> e.g. Population Comparison

Show results to others
=> e.g. 3D plots

Basic concept of a FlowJo analysis

- Load Samples (Workspace)
- Group Samples (Workspace)
- Make Analyses
  - Gate (Graph Window)
  - Specific Analyses (Platform Windows)
  - Statistics (Workspace or Graph Window)
- Make Tables (Table Editor)
- Make Reports (Layout Editor)

Start Live Demo

Prepare data for analysis

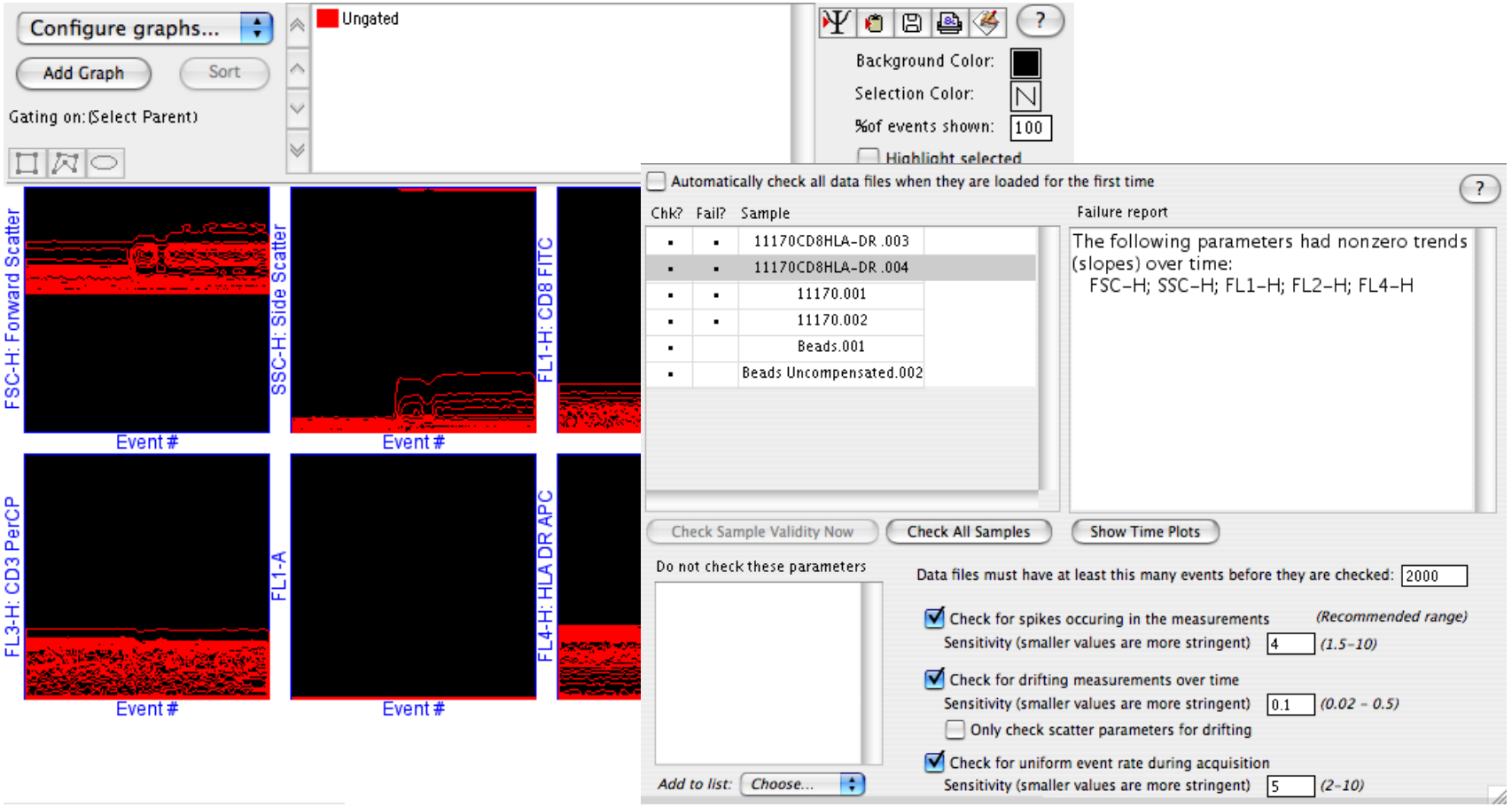
Before you start analysing

Verify that your data is ok

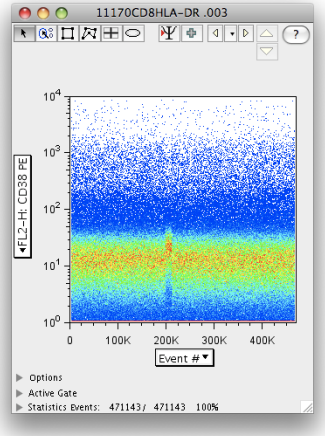
Compensate your data

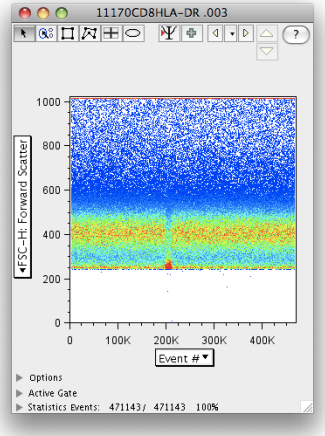
If needed derive new parameters

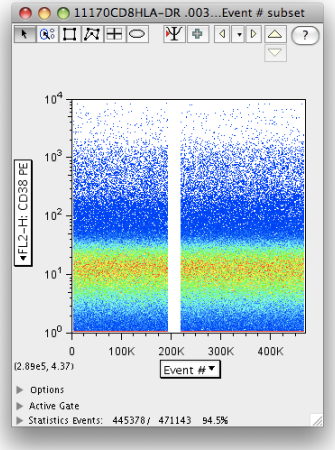
Sample validity verification

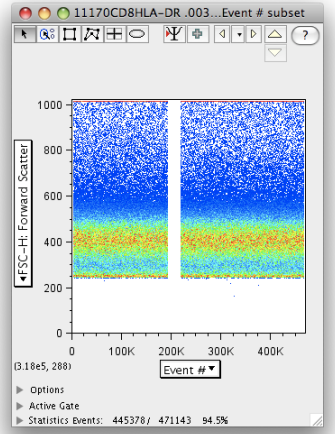


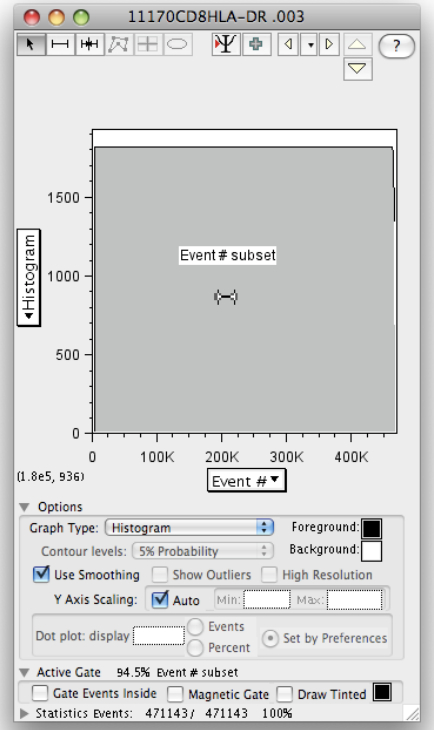
Checking Data for Validity





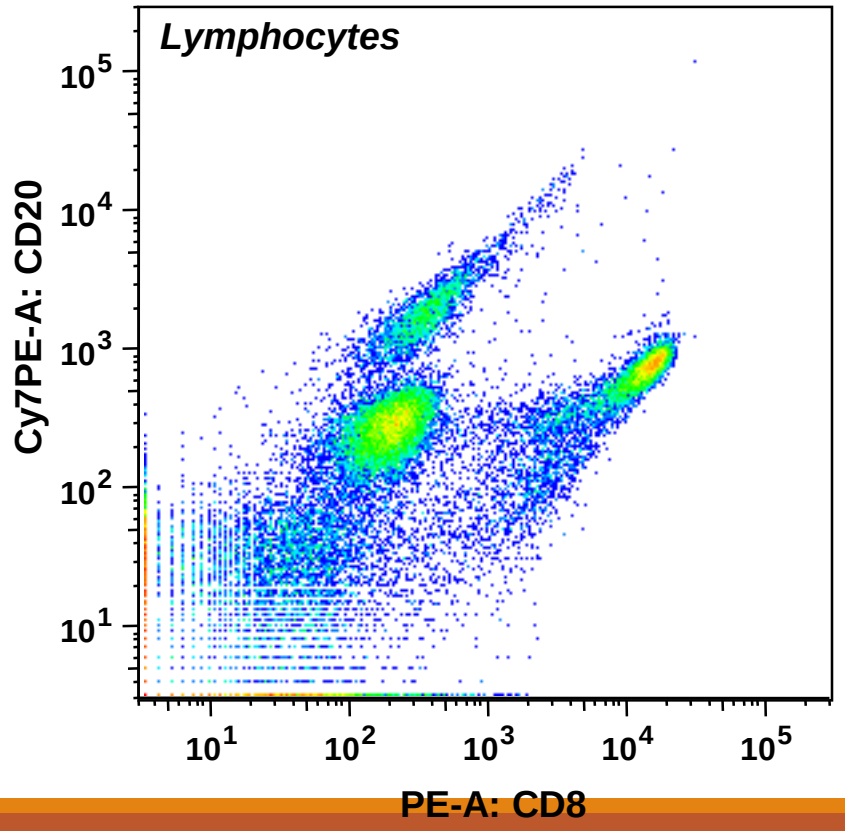




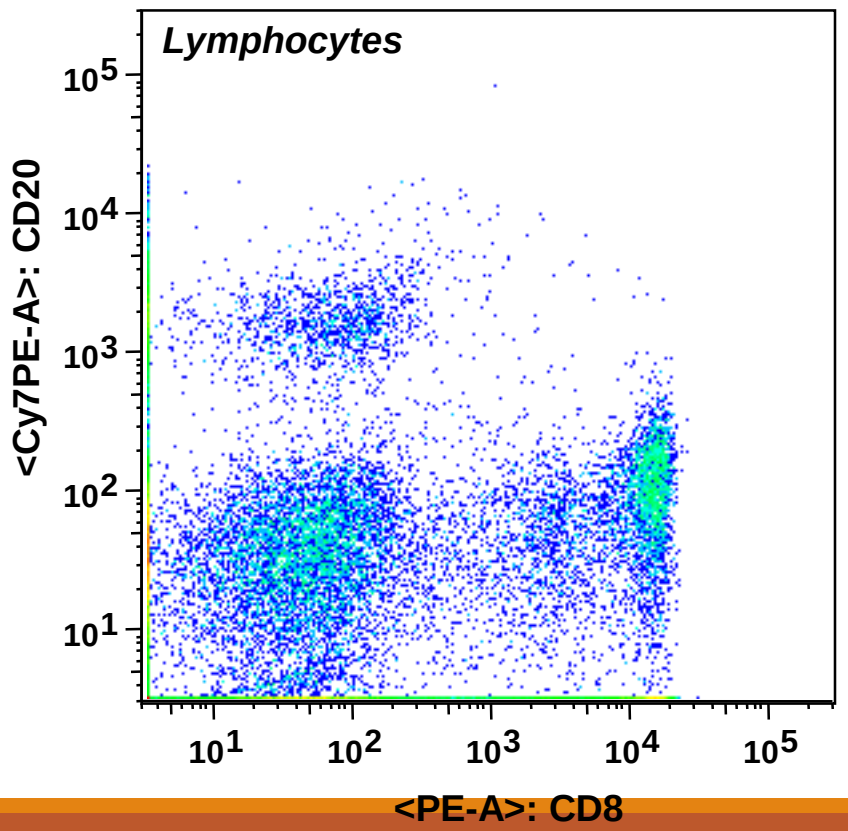


Compensation

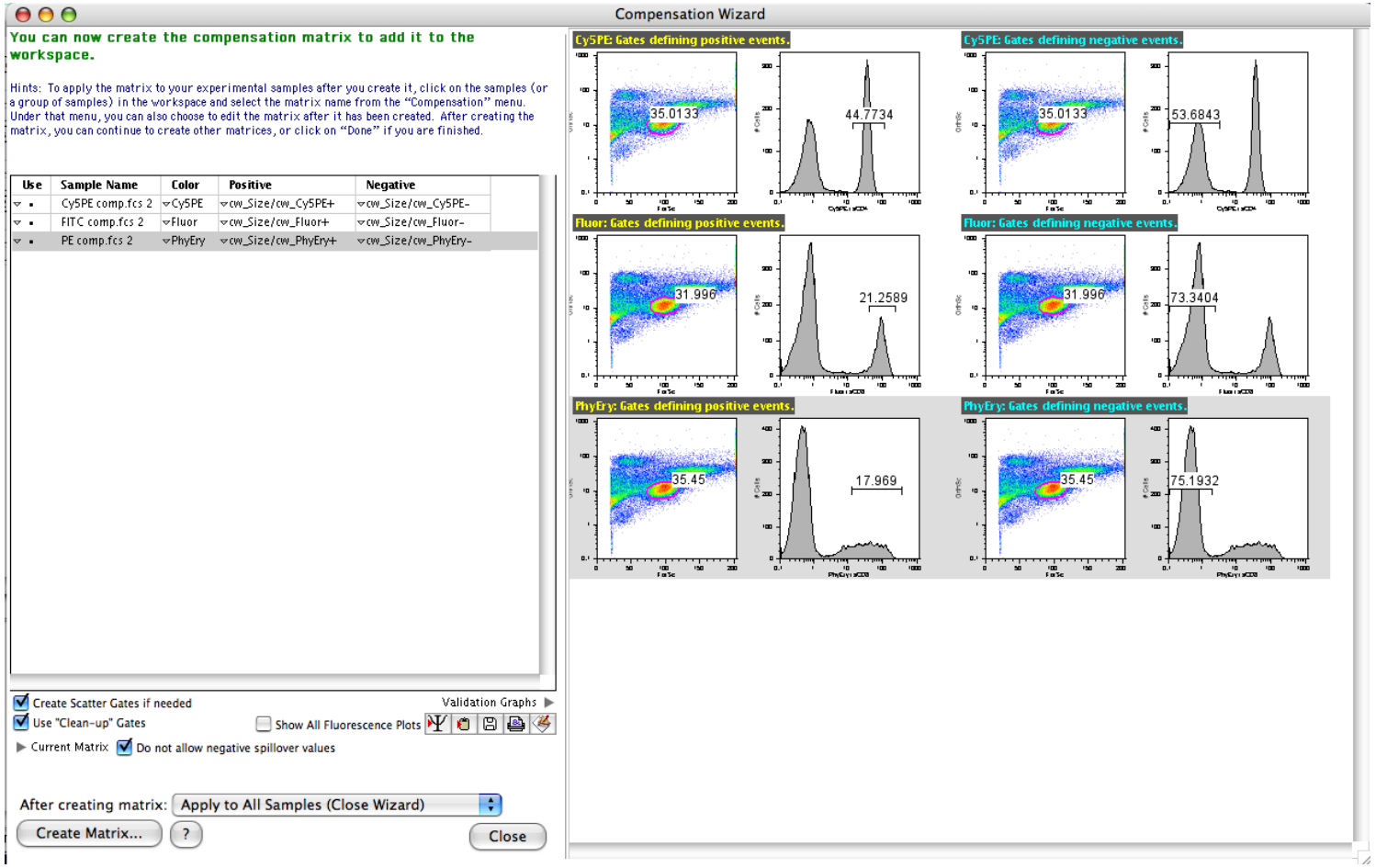
Uncompensated



Compensated



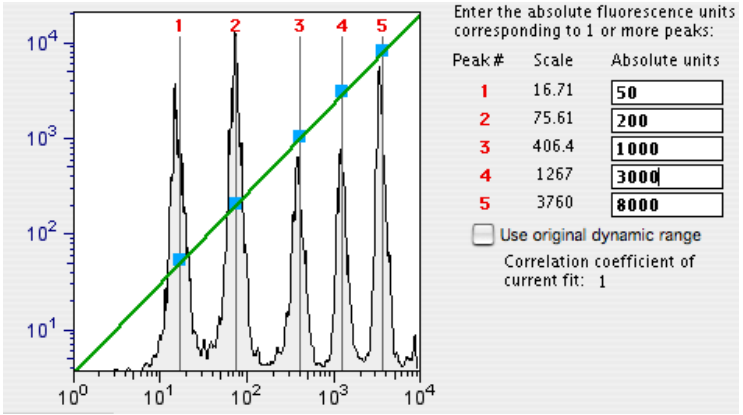
Compensation Wizard

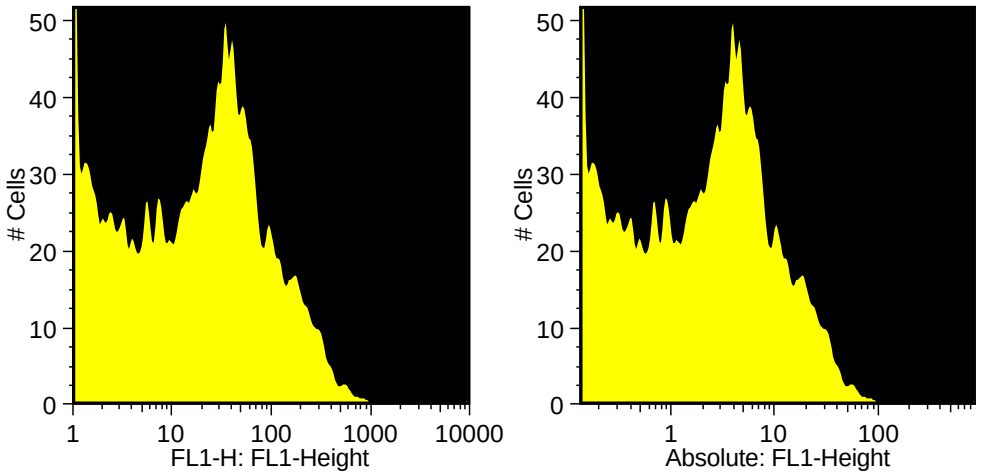


Calibration

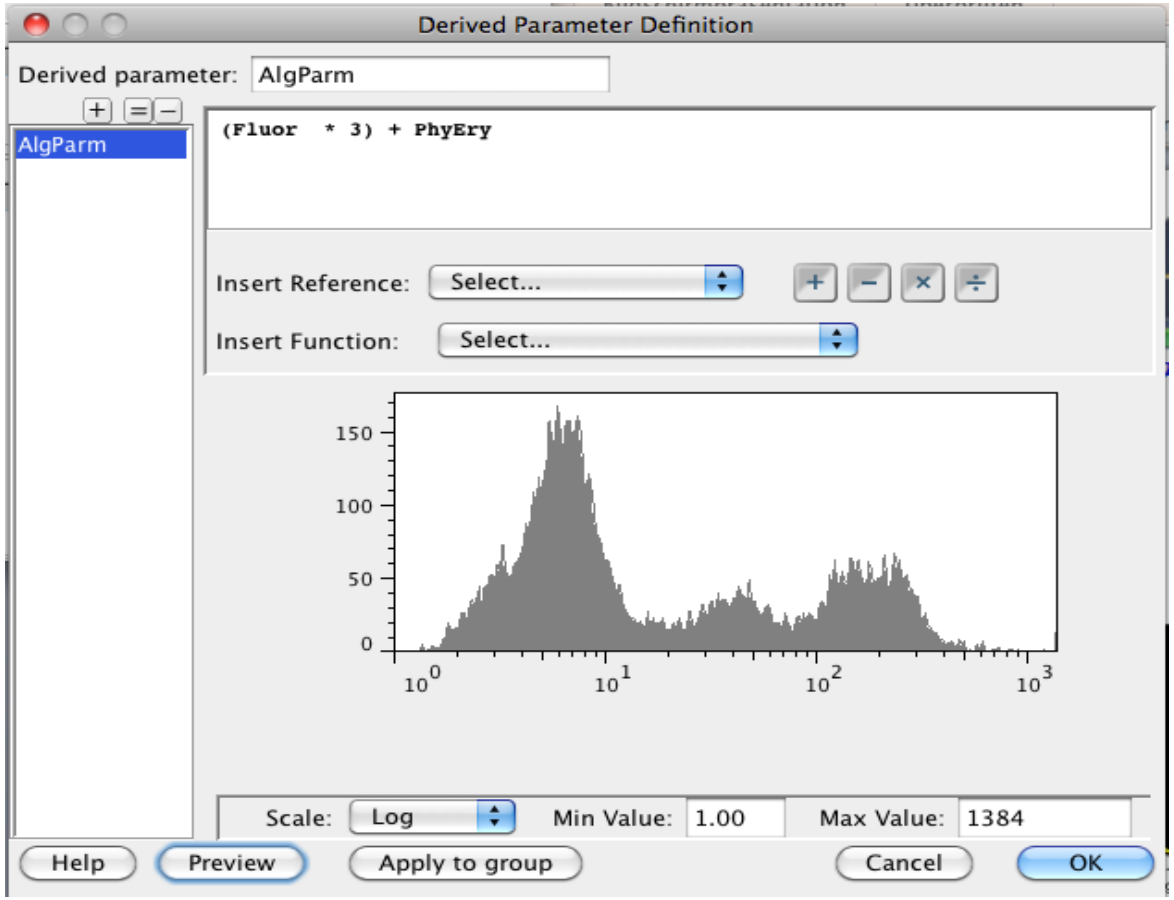
Convert fluorescence measurements into a calibrated unit.

-e.g., absolute number of FITC molecules per cell.



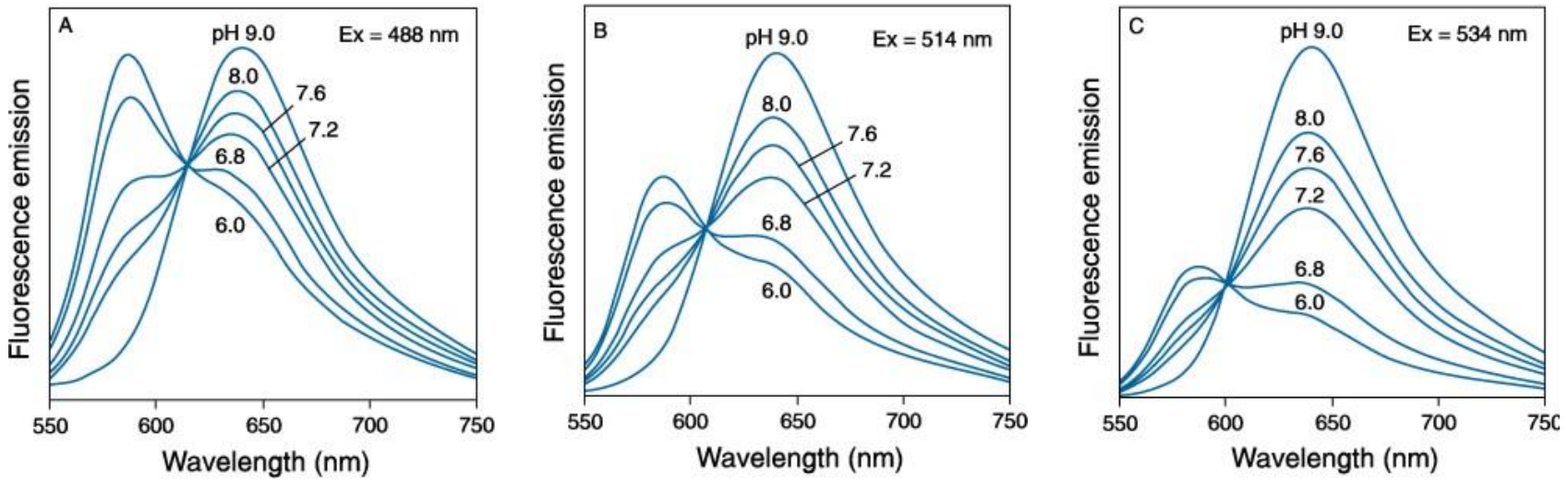


Derived Parameters

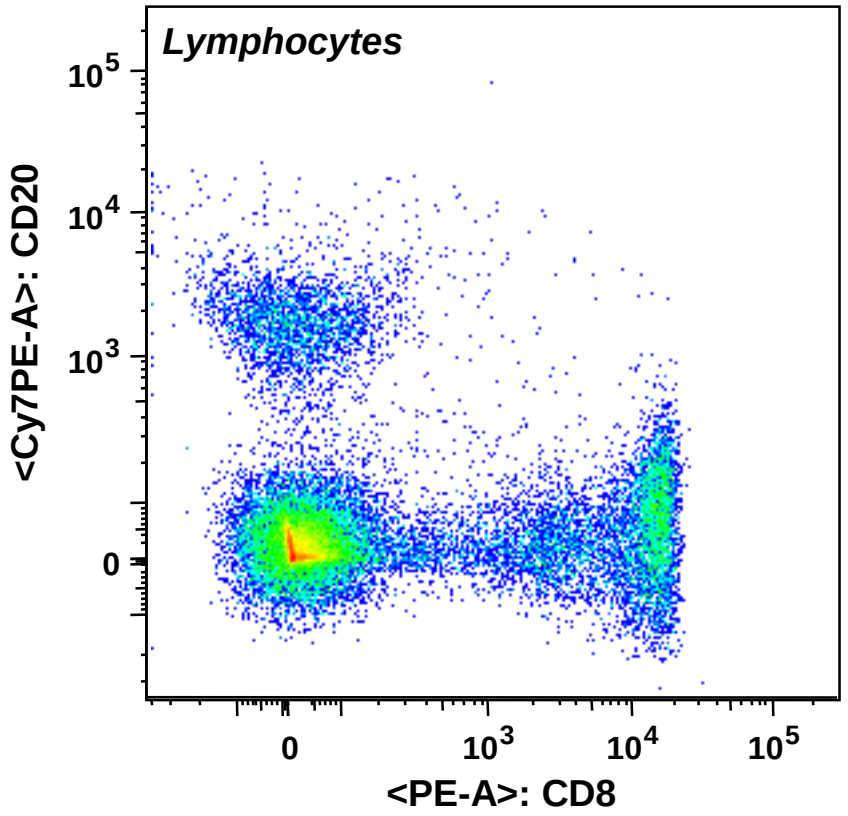


Ratiometric measurments

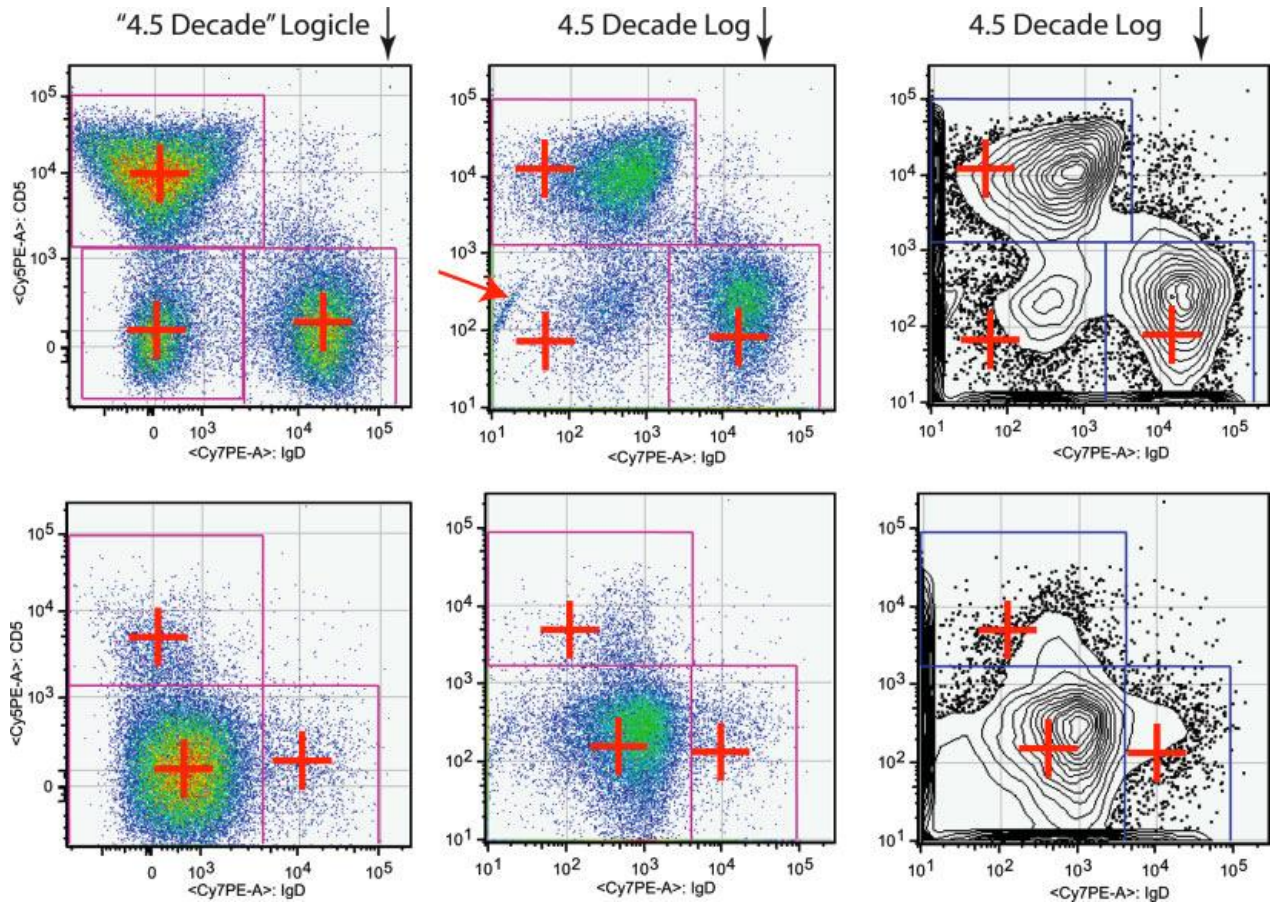
SNARF-1



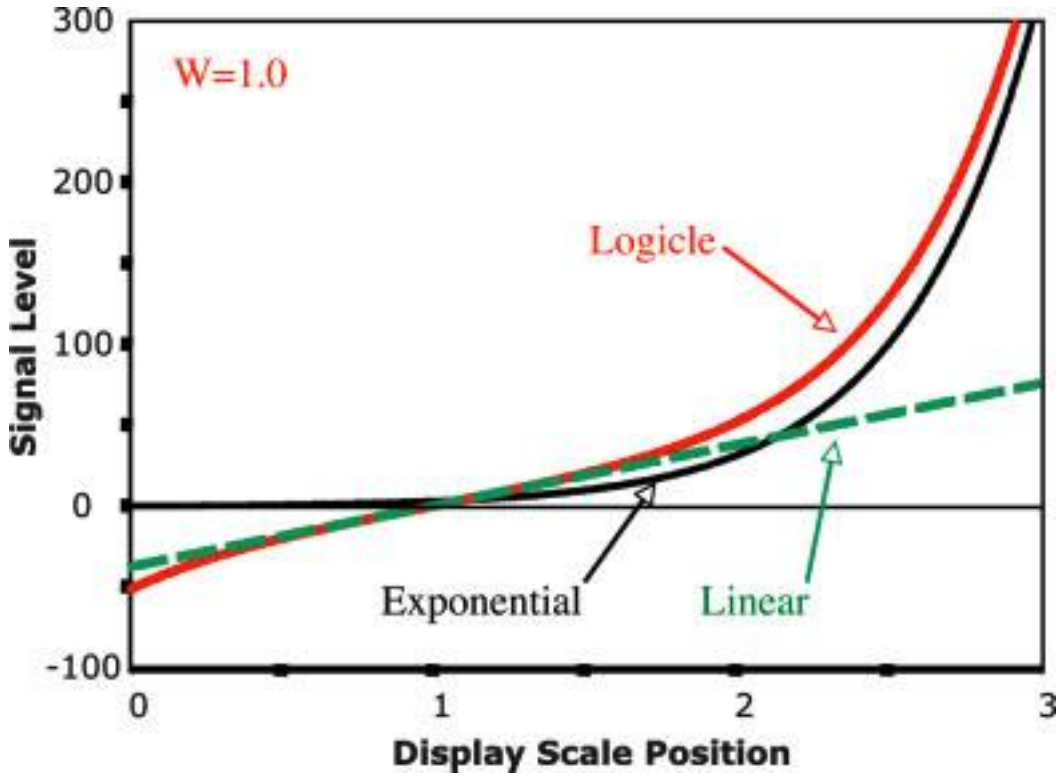
Transformations



‘Logicle’ displays



‘Logicle’ displays



Logicle Scale

Function constructed for FACS data display

Start with the sinh function:

$$\sinh(x) = (e^{x} - e^{-x})/2$$

We generalized this as a biexponential function:

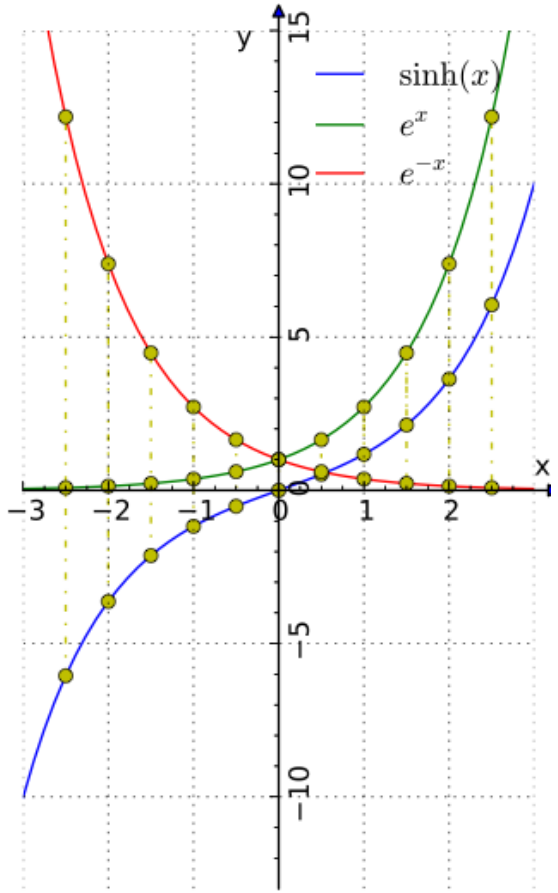
$$v(x; a, b, c, d, k) = ae^{bx} - ce^{-dx} + k$$

Our specifications and constraints (V and V''= 0 at x=0) lead to:

$$V = a(e^{x} - p^{2}e^{-x/p} + p^{2} - 1))$$

where V is the data value to be plotted at display position x in the plot, a is a scaling factor and p is the strength of the linearization. We have called this the "Logicle" function.

sinh



Logicle Scale

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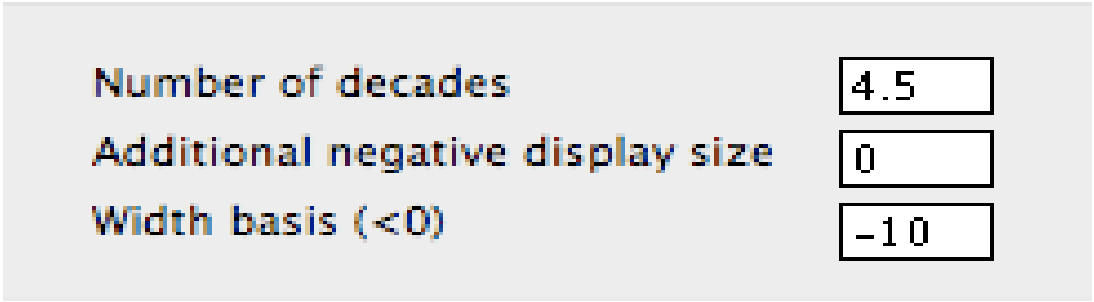
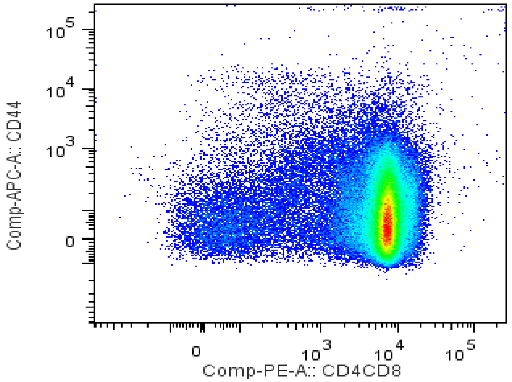
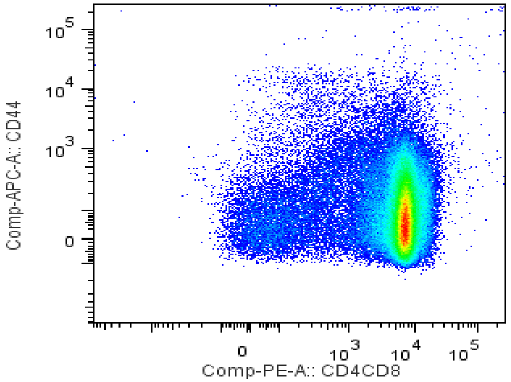
where V is the data value to be plotted at display position x in the plot, a is a scaling factor and p is the strength of the linearization. We have called this the "Logicle" function.

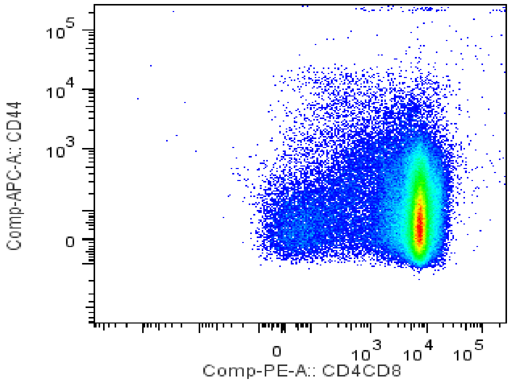
Transformation parameters



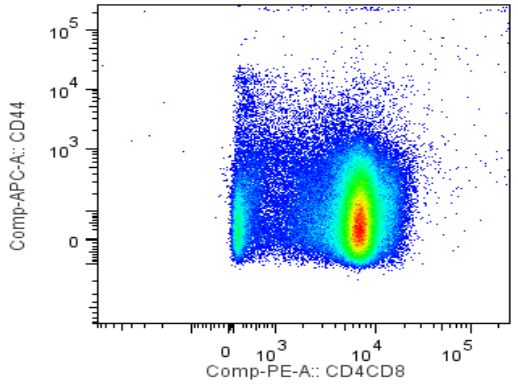
Extra negative decades



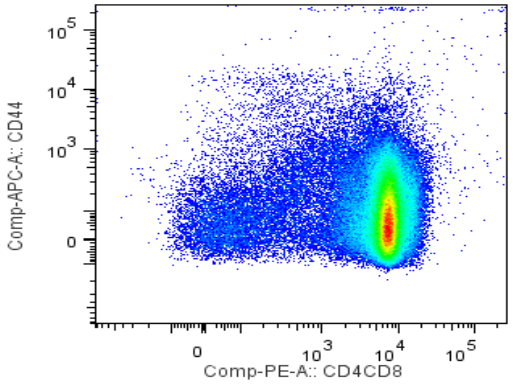




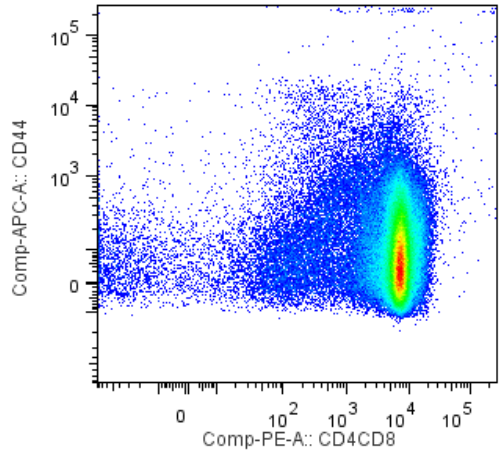
Positive decades



3.5

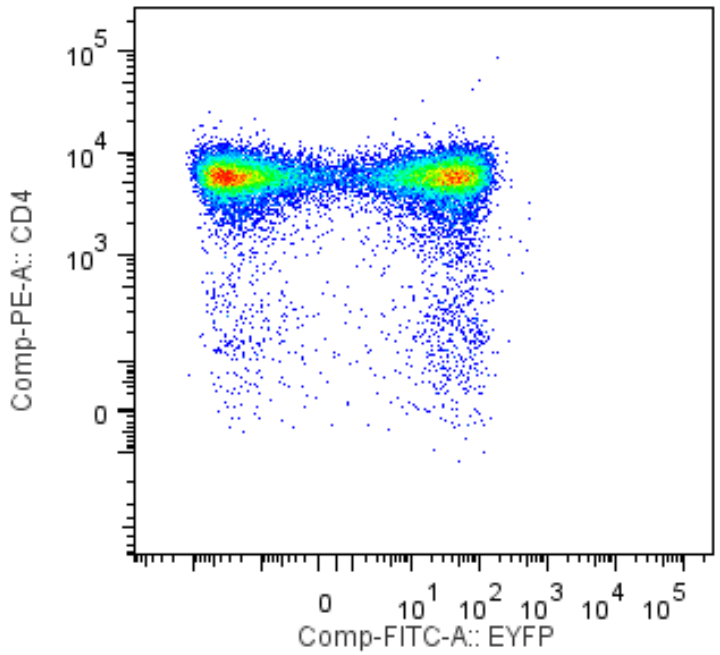


4.5

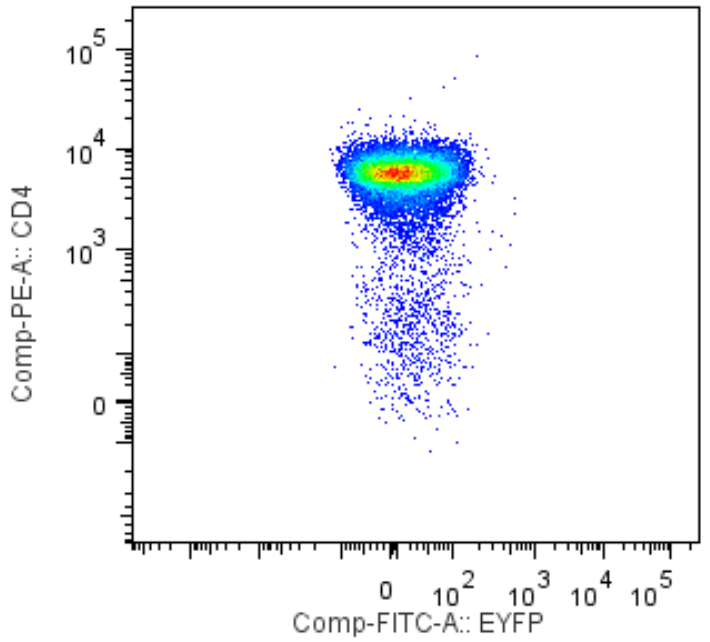


5.5

Width

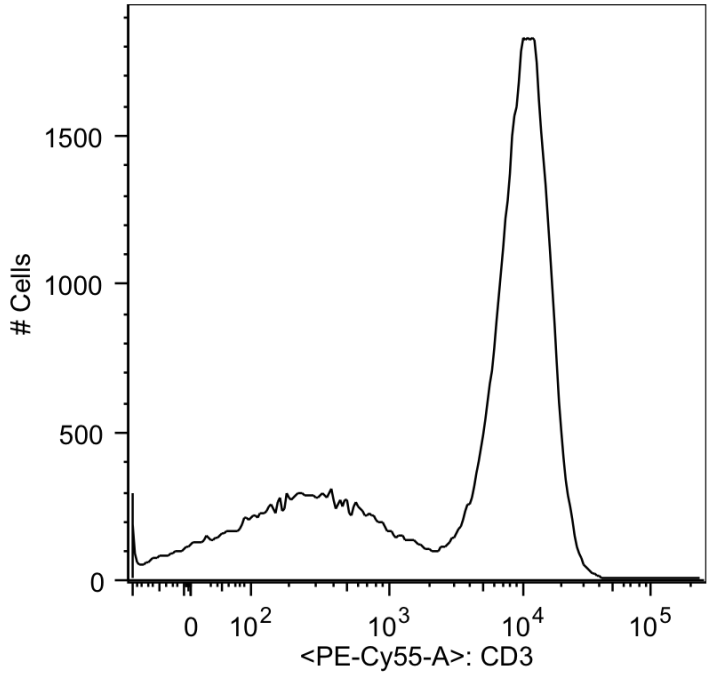


Width = -2

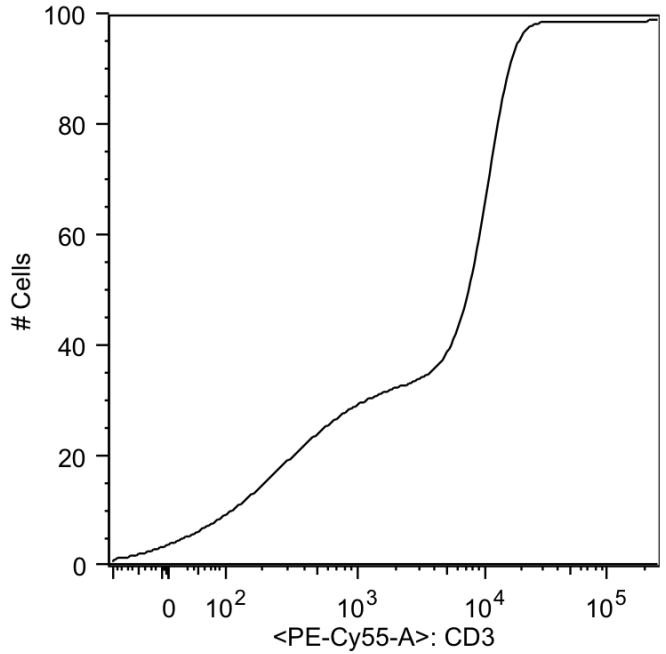


Width = -100

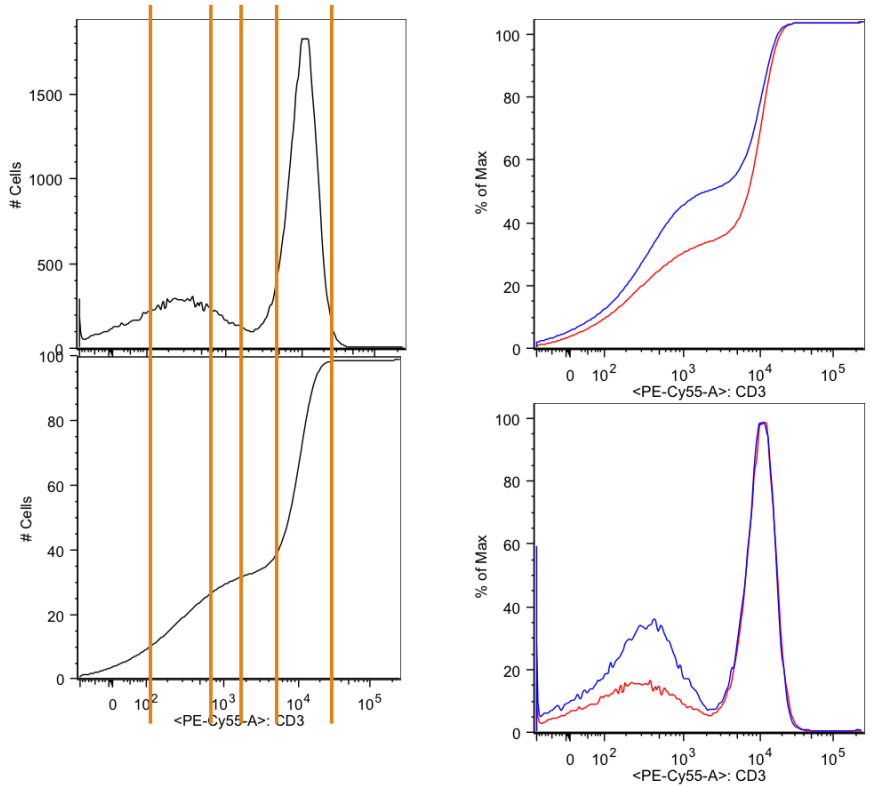
Univariate Plot Types



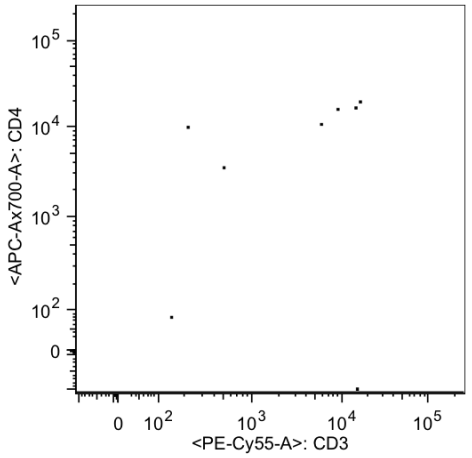
Histogram



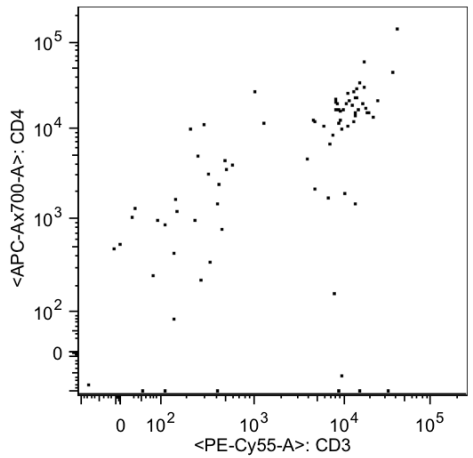
CDF
(Cumulative Distribution Function)



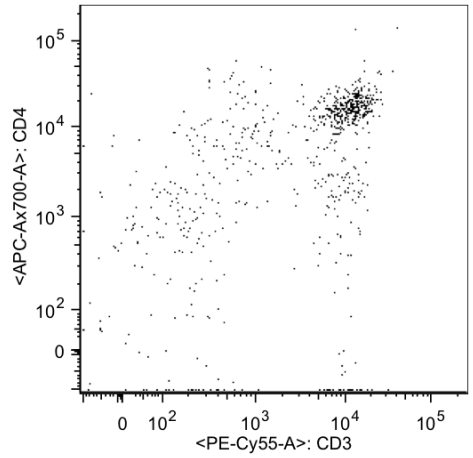
Bivariate Plot Types: Dot Plots



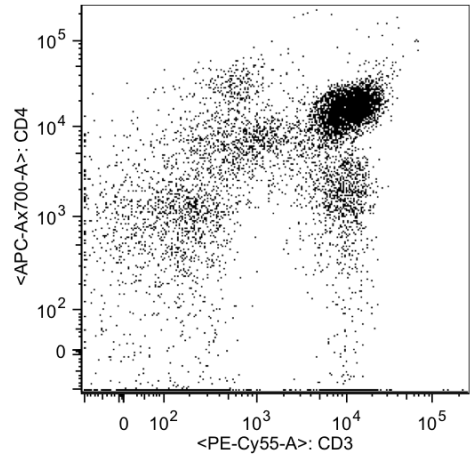
Low Resolution
8 events



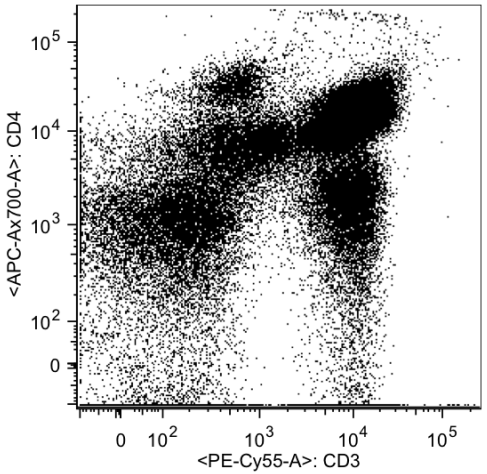
Low Resolution
80 events



High Resolution
800 events

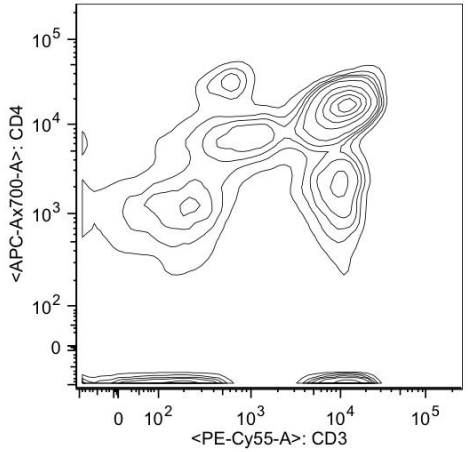


High Resolution
8000 events

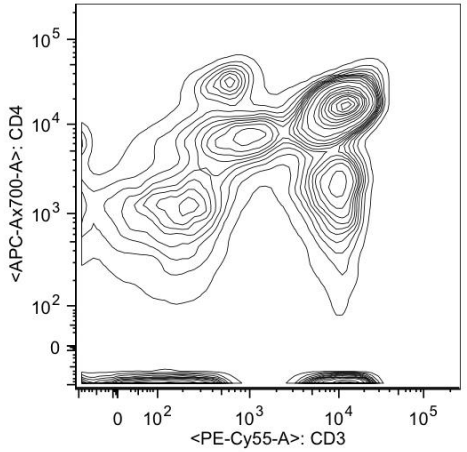


High Resolution
80000 events

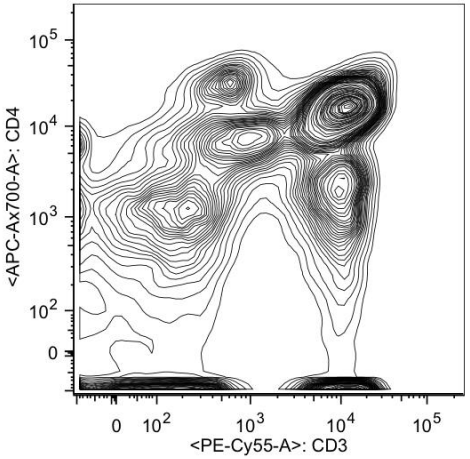
Bivariate Plot Types: Contour Plots



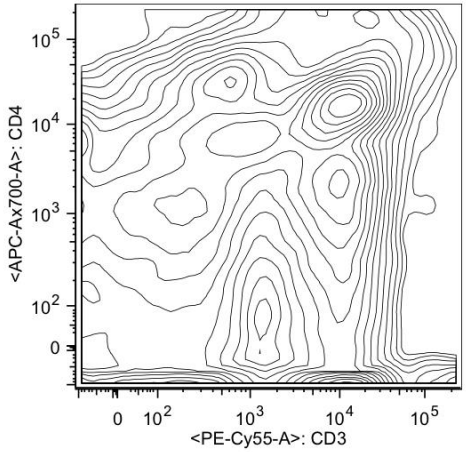
10% Probability
10 surfaces
9 borders



5% Probability
20 surfaces
19 borders



2% Probability
50 surfaces
29 borders

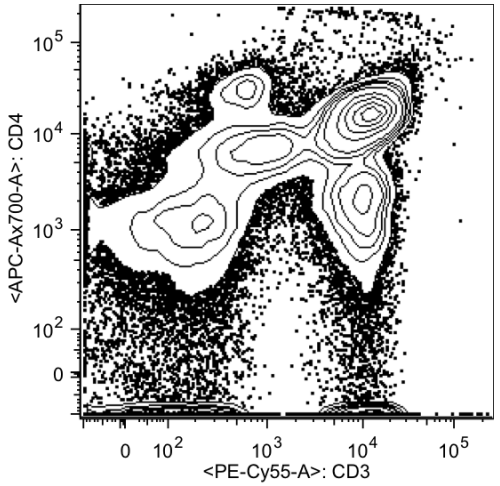


Logarithmic 50%
surfaces and borders
depend on cell number

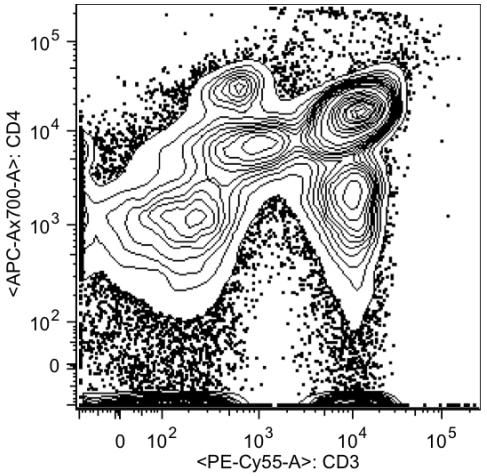
Each surface same probability.

2 (Log50(1.69897)?) times more
cells per surface

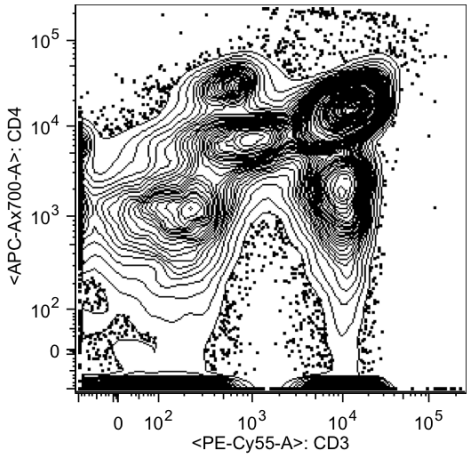
Bivariate Plot Types: Contour Plots with outliers



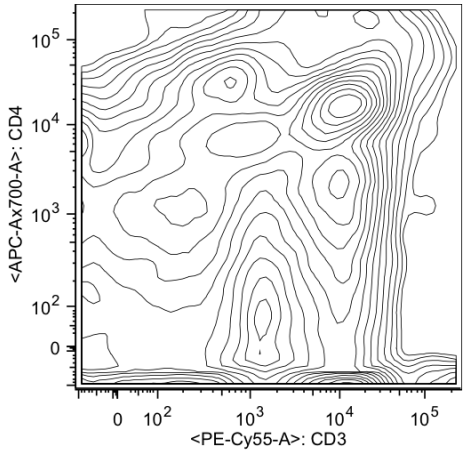
10% Probability
10 surfaces
9 borders



5% Probability
20 surfaces
19 borders



2% Probability
50 surfaces
29 borders

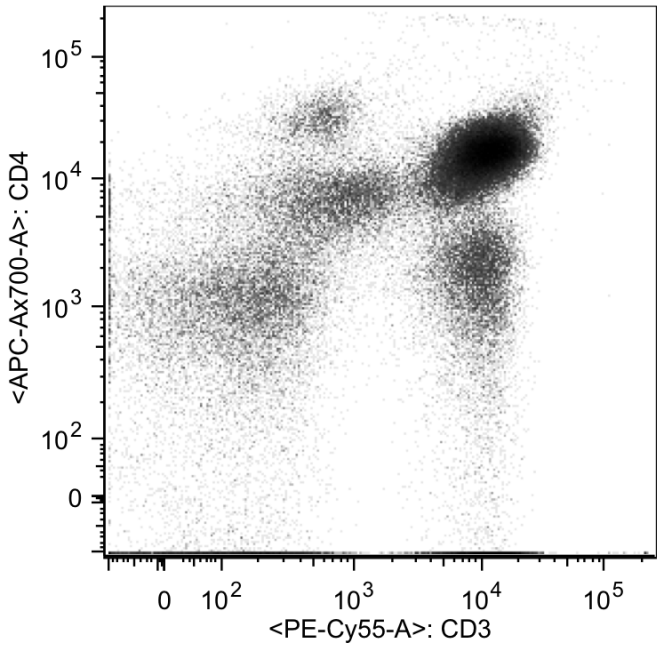


Logarithmic 50%
surfaces and borders
depend on cell number

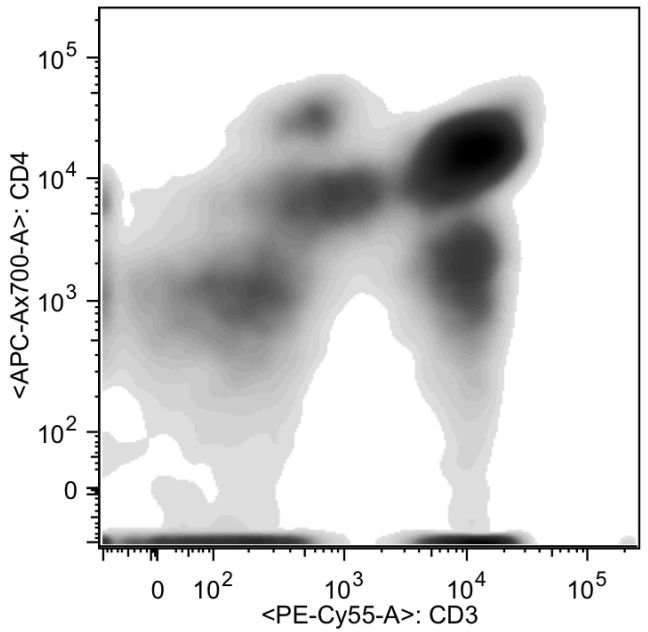
Each surface same probability.

2 (Log50(1.69897)?) times more
cells per surface

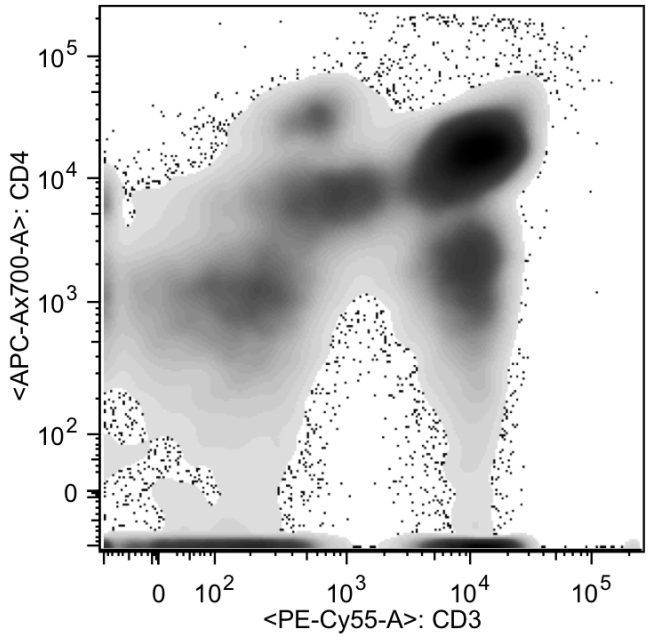
Bivariate Plot Types: Density Plots



Without smoothing

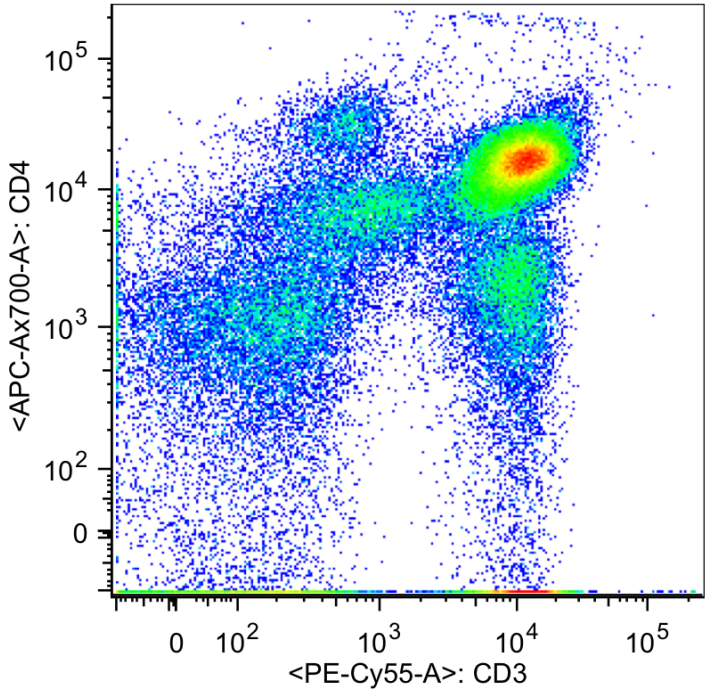


With smoothing

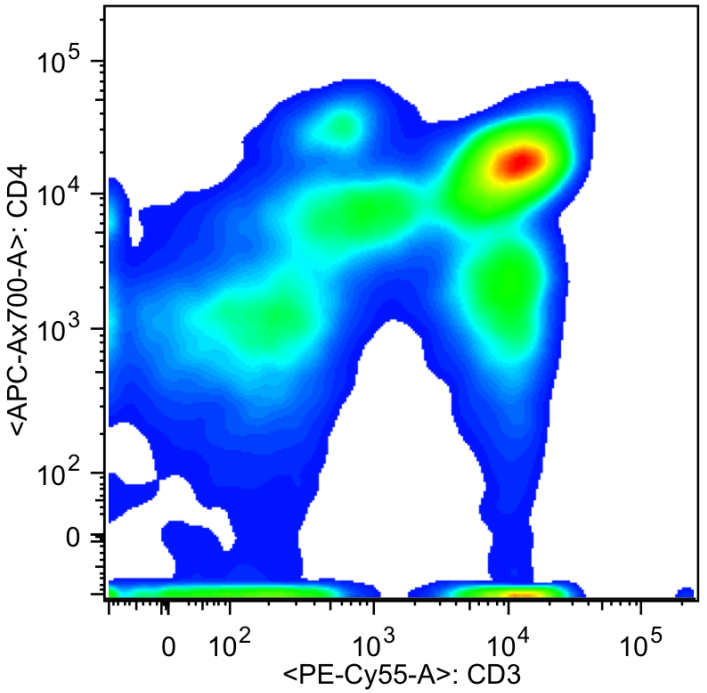


With smoothing and outliers

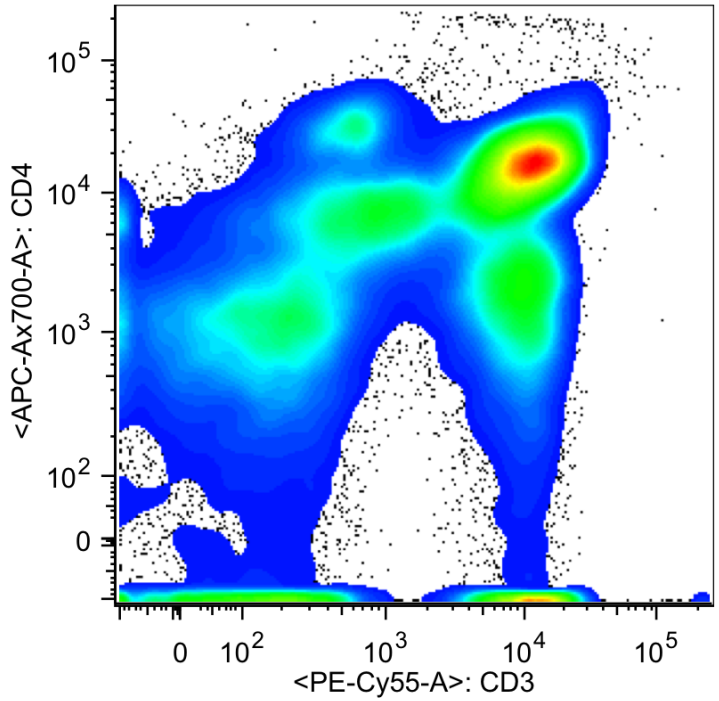
Bivariate Plot Types: Pseudo-color Plots



Without smoothing

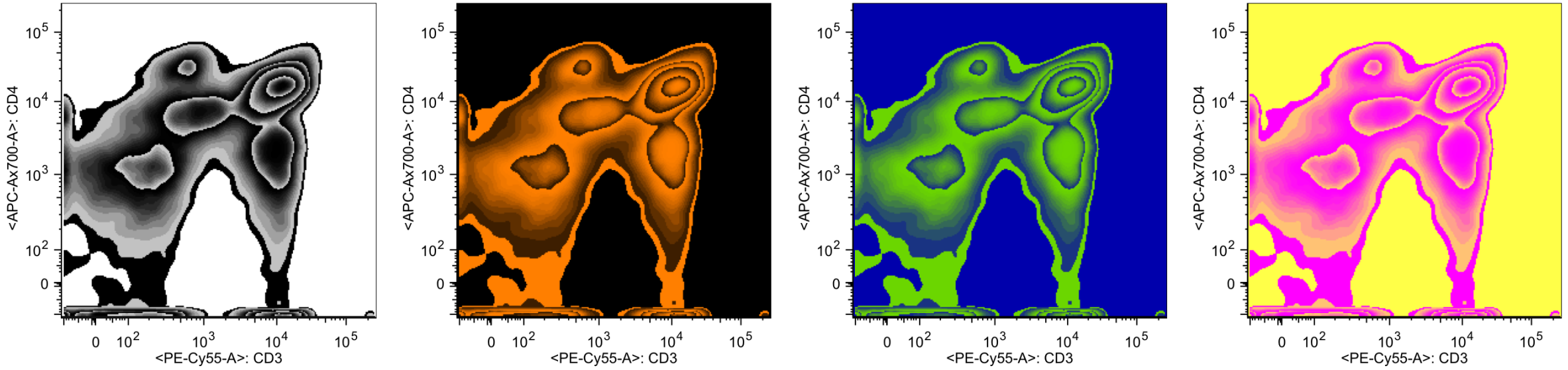


With smoothing

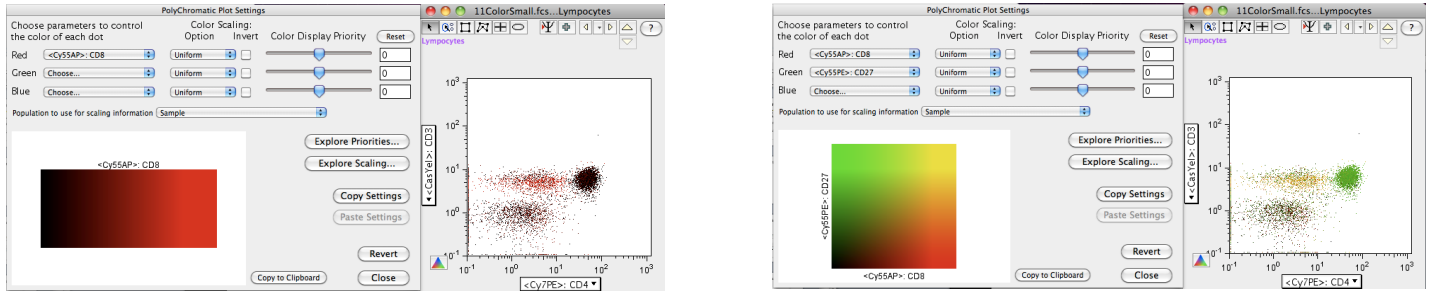


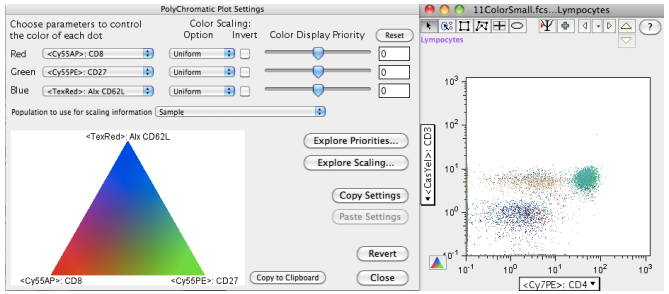
With smoothing and outliers

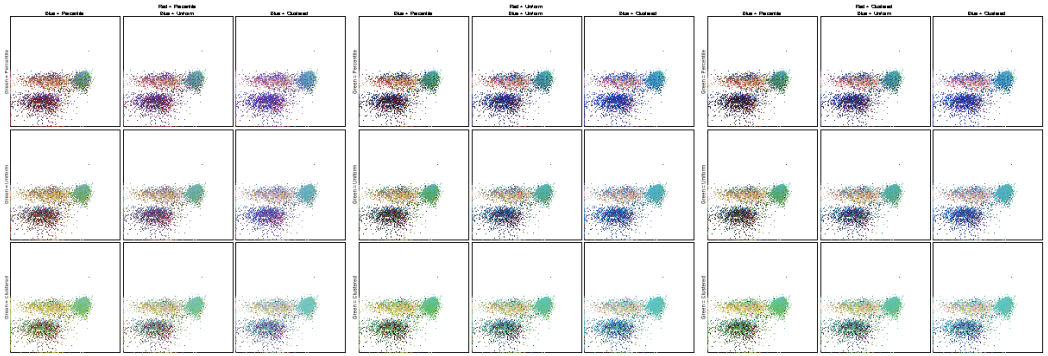
Bivariate Plot Types: Zebra Plots



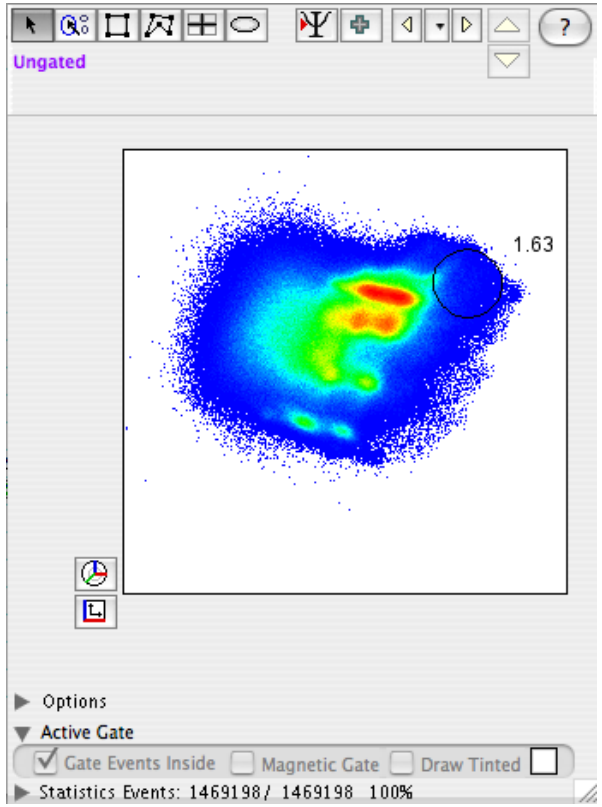
Polychromatic Plots

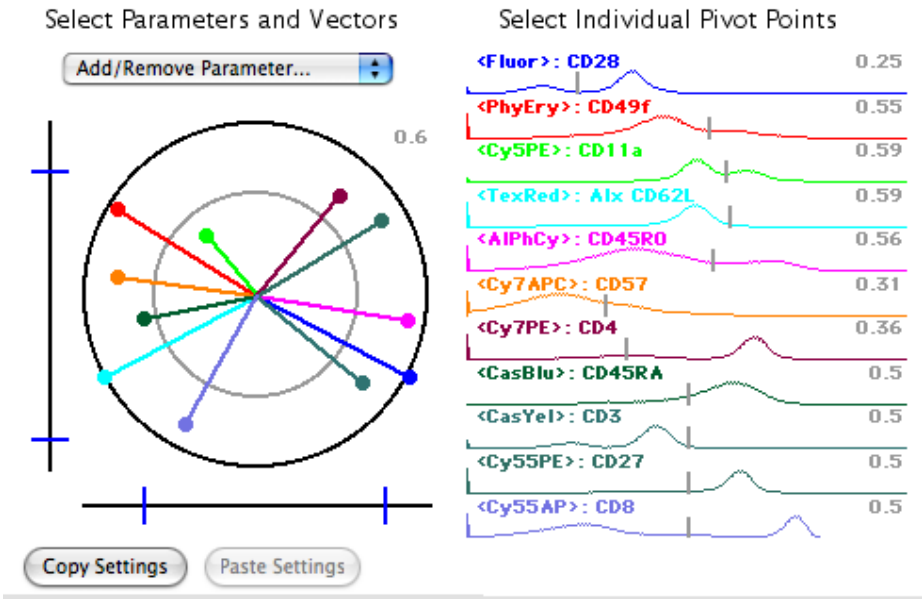




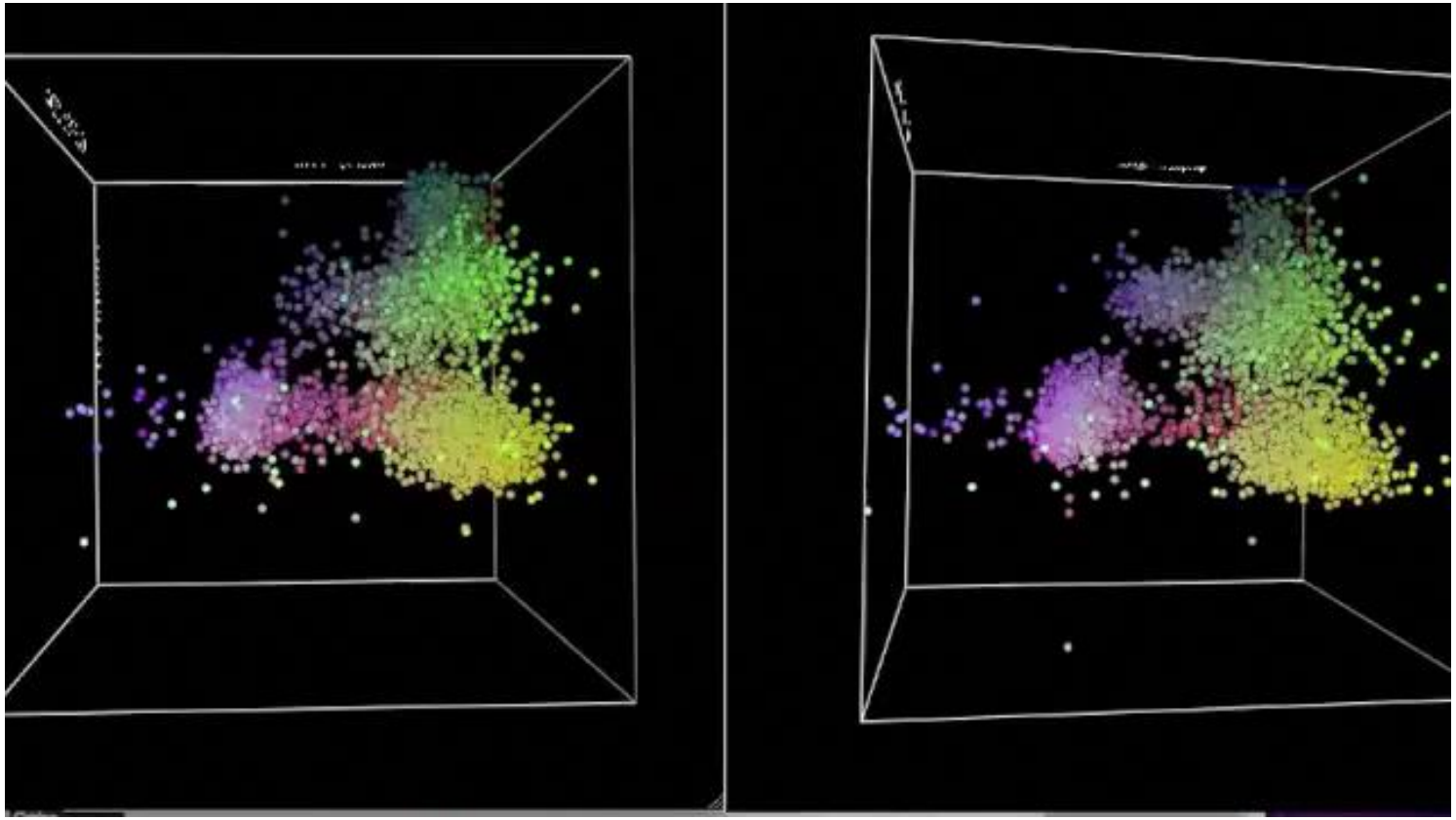


Polyvariate Plot

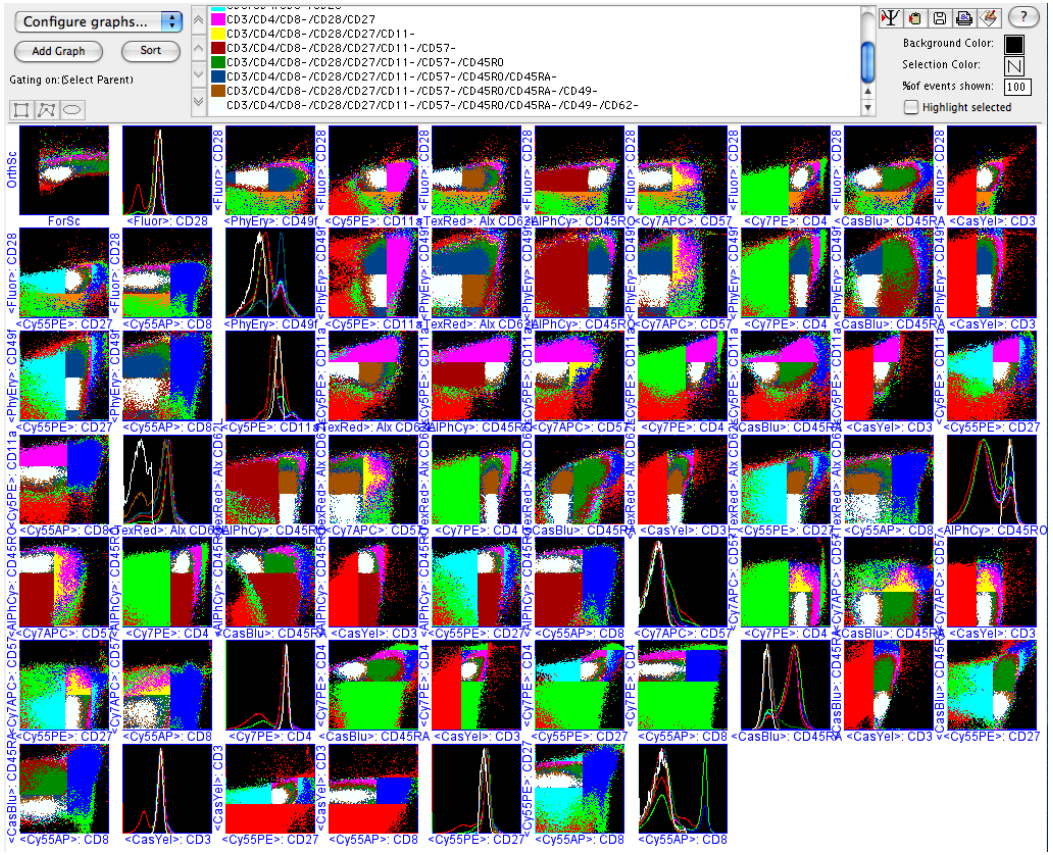




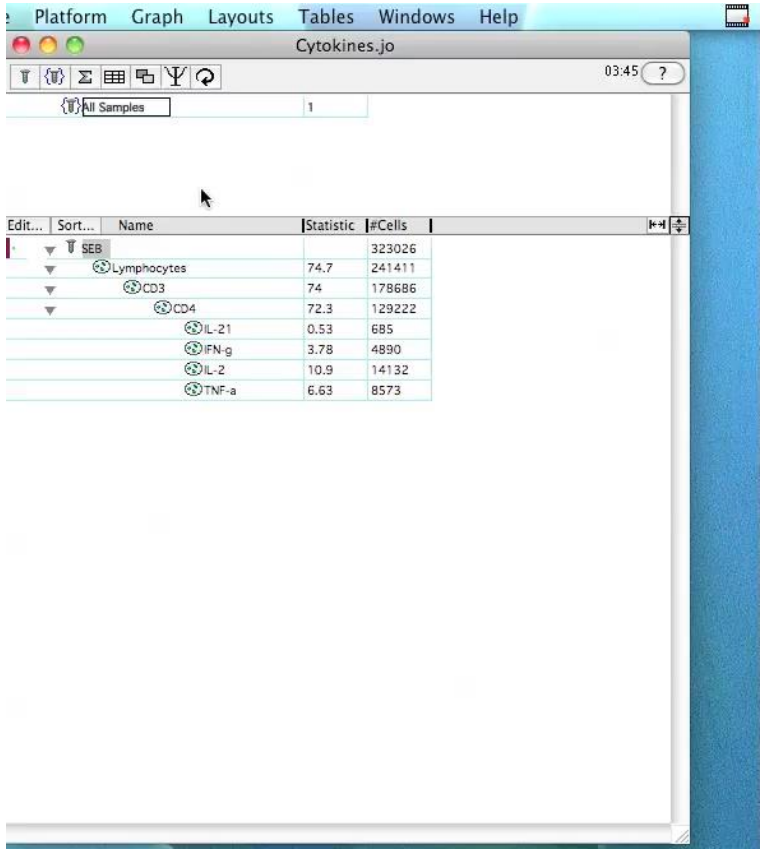
3D presentation



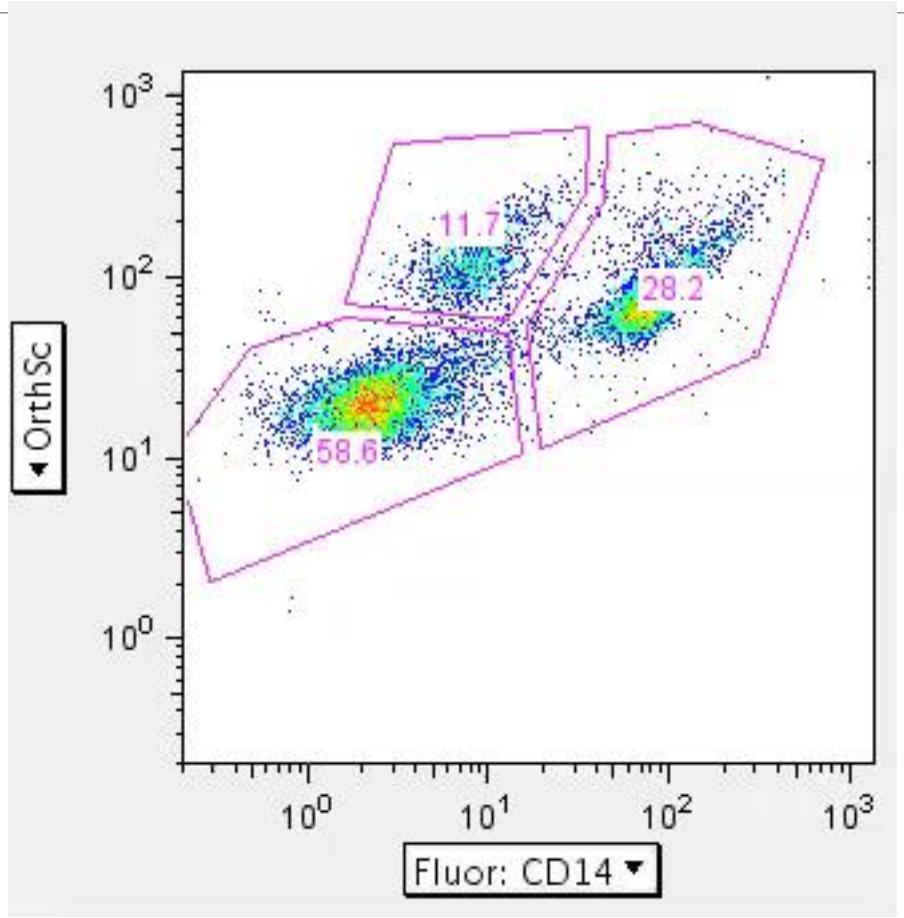
Multigraph overlay



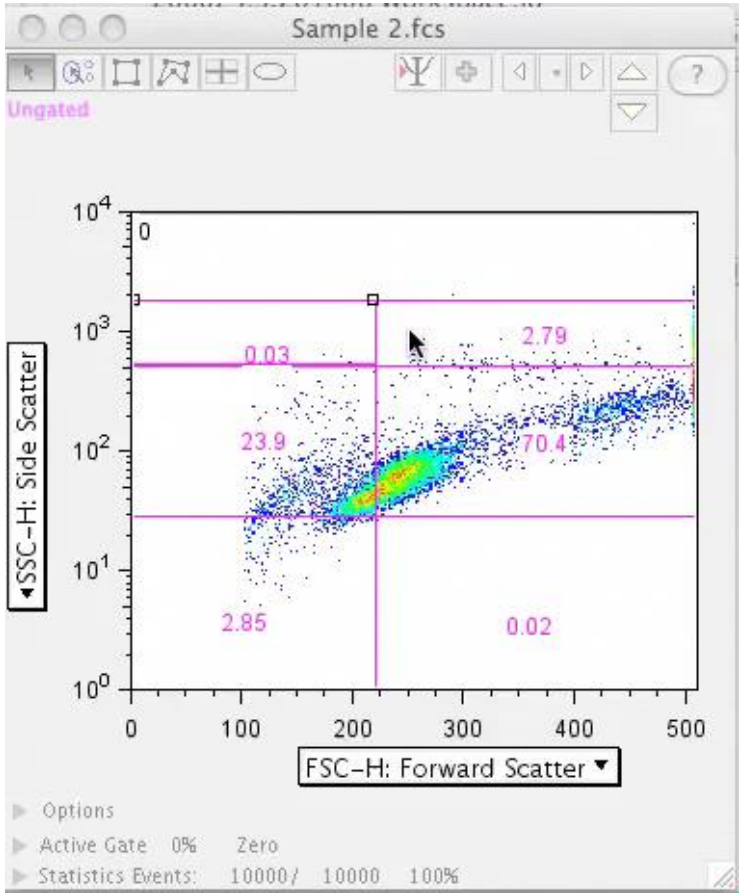
Combination Gates



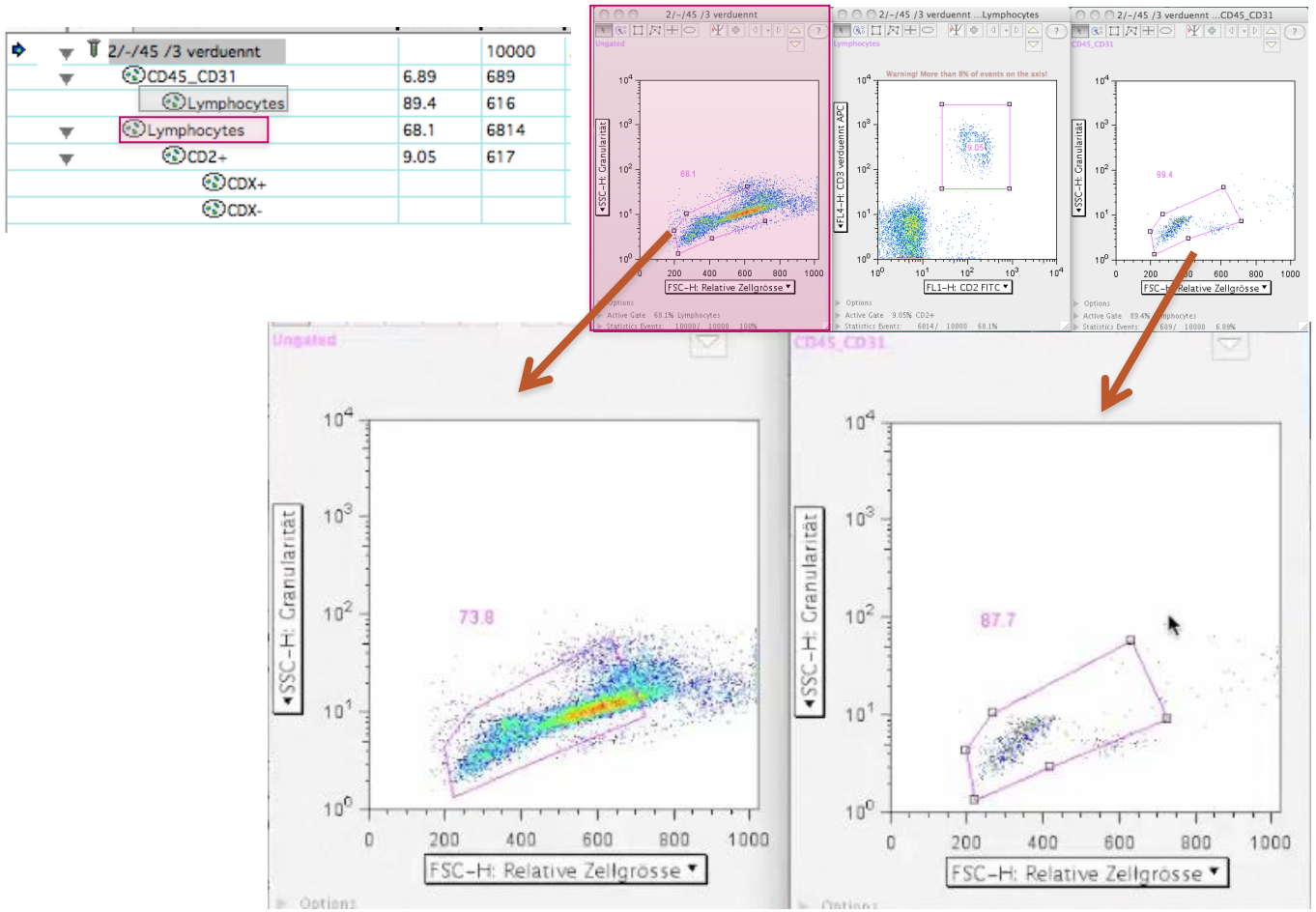
Gate molding



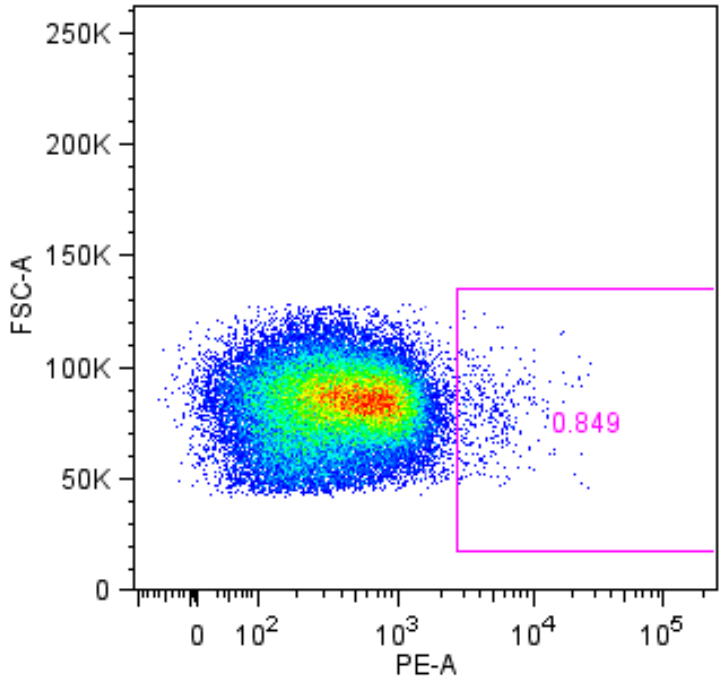
FJML

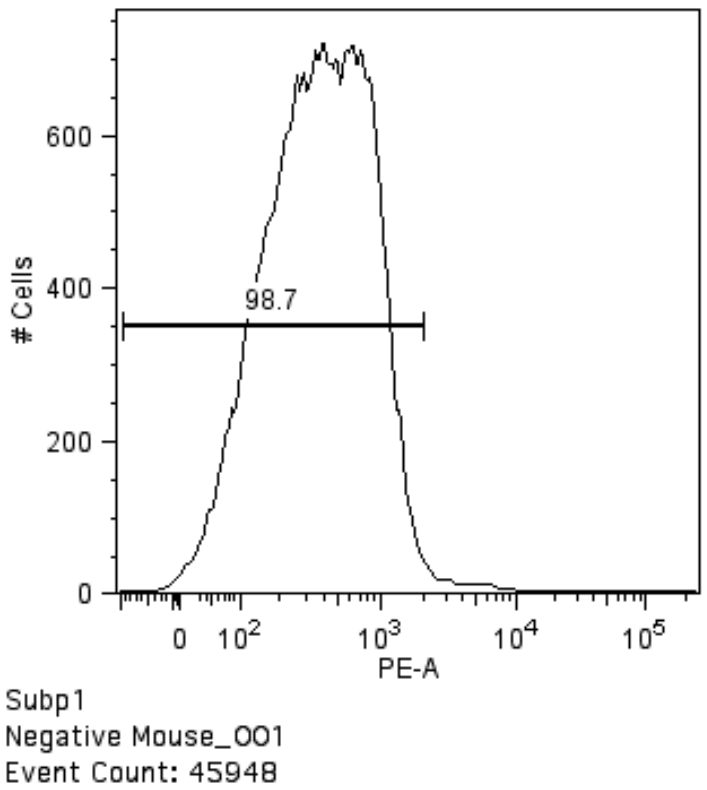

Sample 2.fcs
Ungated
0
0.03
2.79
23.9
70.4
2.85
0.02
SSC-H: Side Scatter
FSC-H: Forward Scatter
Options
Active Gate 0% Zero
Statistics Events: 10000/ 10000 100%

FJML


2/-/45 /3 verduennt
10000
CD45_CD31
6.89
689
Lymphocytes
89.4
616
Lymphocytes
68.1
6814
CD2+
9.05
617
CDX+
CDX-
2/-/45 /3 verduennt
2/-/45 /3 verduennt ...Lymphocytes
2/-/45 /3 verduennt ...CD45_CD31
Ungated
CD45_CD31
SSC-H: Granularität
FSC-H: Relative Zellgrösse
FL1-H: CD2 FITC
FL4-H: CD3 verduennt APC
73.8
87.7
Warning! More than 8% of events on the axis!

FJML


250K
200K
150K
100K
50K
0
FSC-A
0.849
0
10^2
10^3
10^4
10^5
PE-A
Subp1
Negative Mouse_001
Event Count: 45948


600
400
200
0
Cells
98.7
0
10^2
10^3
10^4
10^5
PE-A
Subp1
Negative Mouse_001
Event Count: 45948

Statistics

Get statistics

What kind of statistics can you get?

Frequency

Count

Frequency of Total

Frequency of Parent

Frequency of Grandparent

Frequency of ...

Mean

Median

Mean (Arithmetic Mean)

Geometric Mean

Percentile

Mode

What is MFI?

Deviation

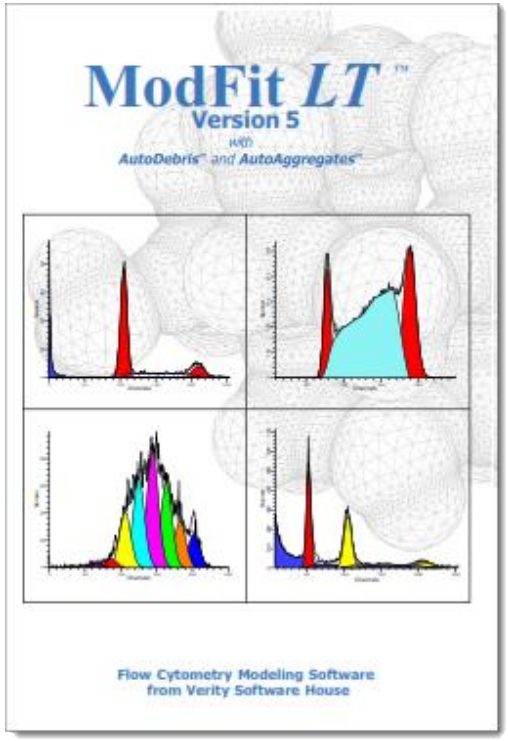
SD (Standard Deviation)

CV (Coefficient of Variation)

Robust CV

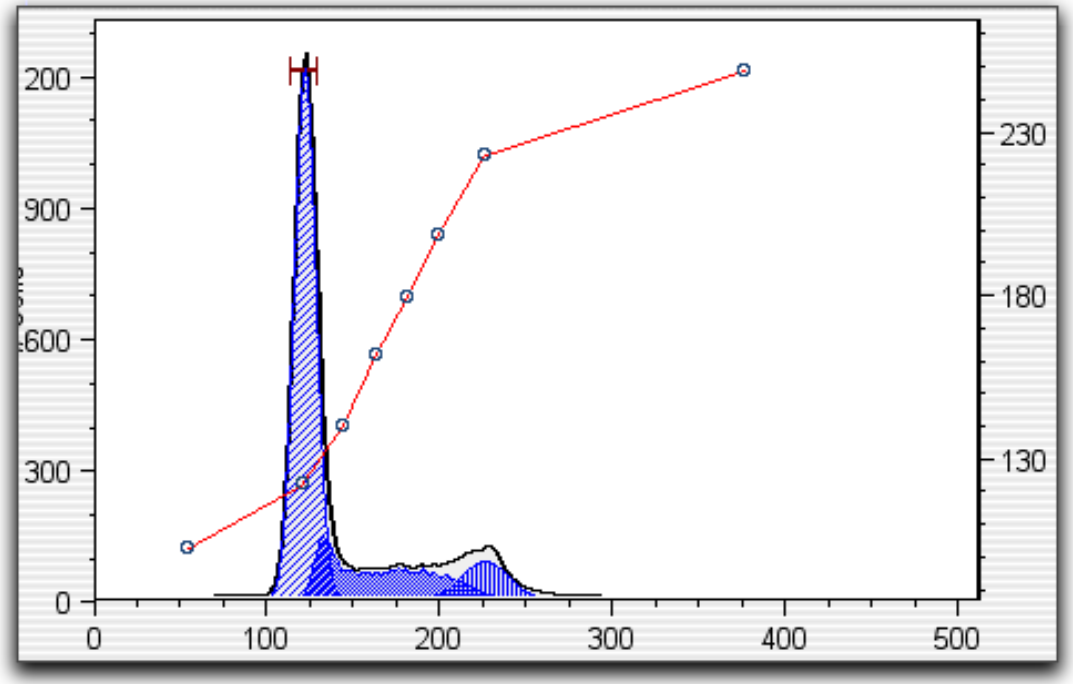
Median Absolute Deviation (%)

Cell Cycle


ModFit LT™
Version 5
with
AutoDebris™ and AutoAggregates™
Flow Cytometry Modeling Software
from Verity Software House

Cell Cycle

View several different models simultaneously and automatically calculate the percentage of cells in G1, S, or G2

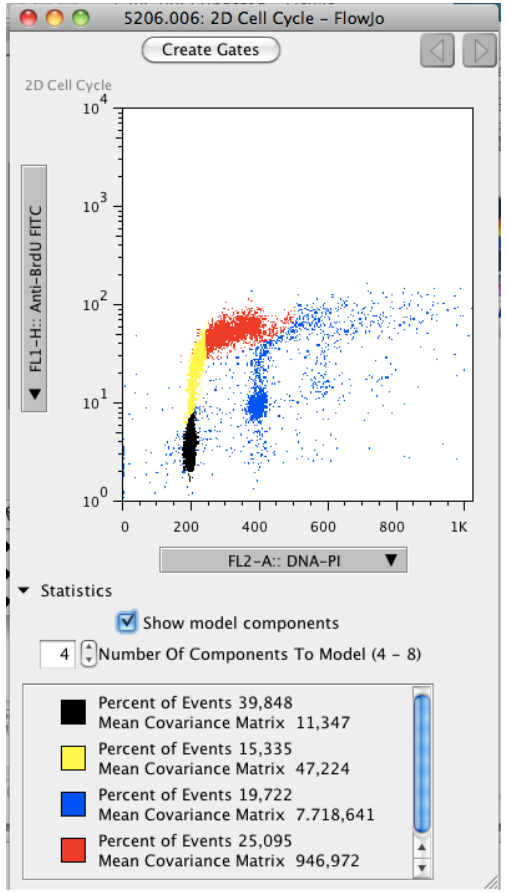


Cells

Median GFP fluorescence

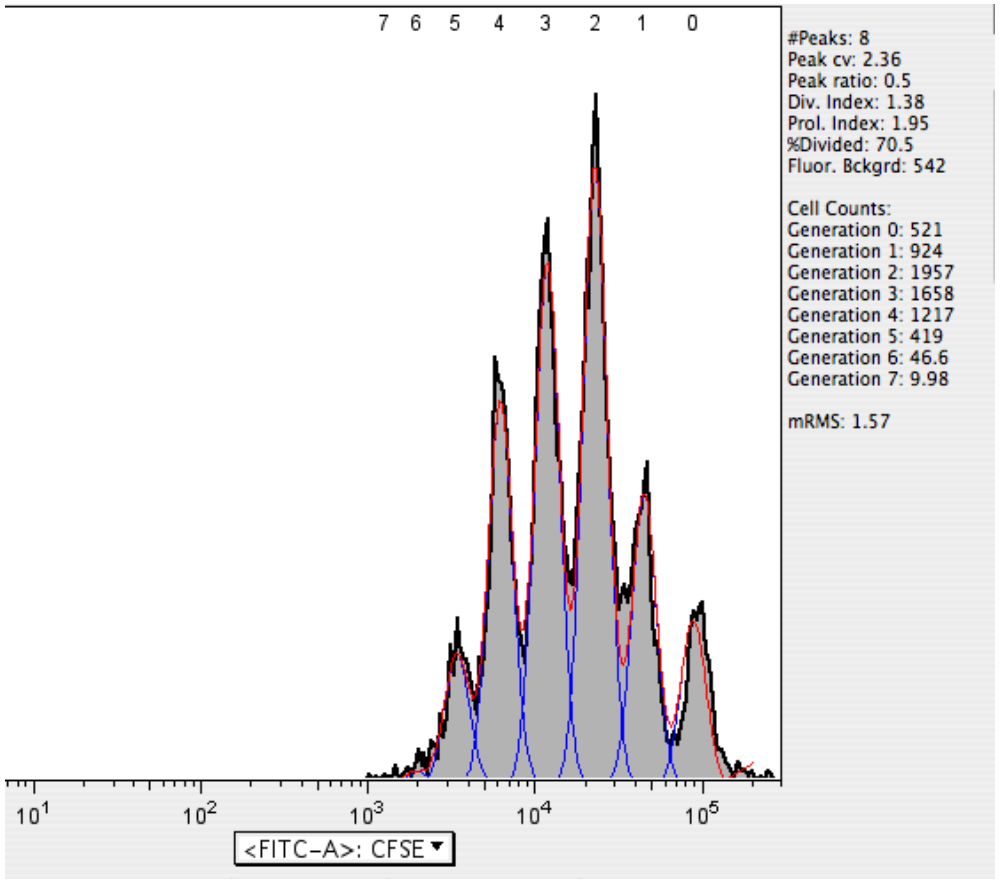
DNA (PI)

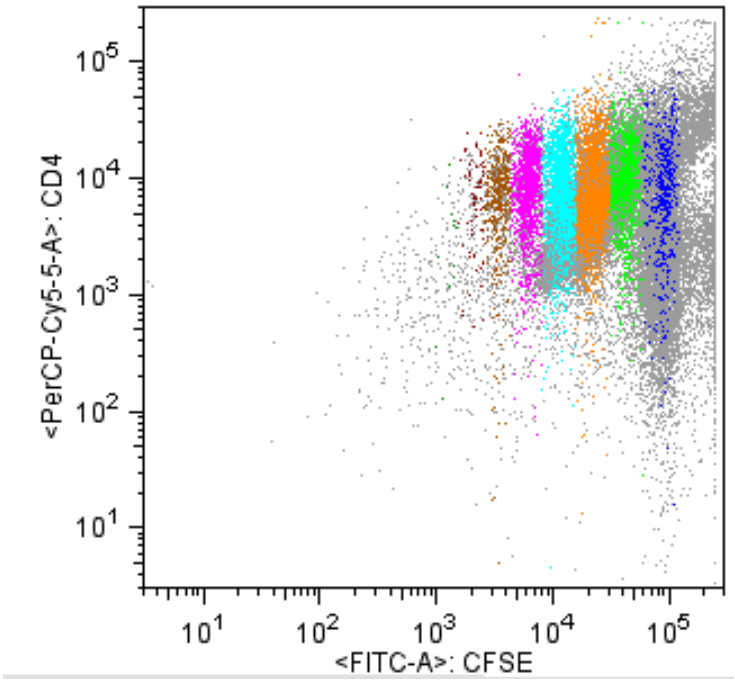
2d Cell Cycle



Proliferation

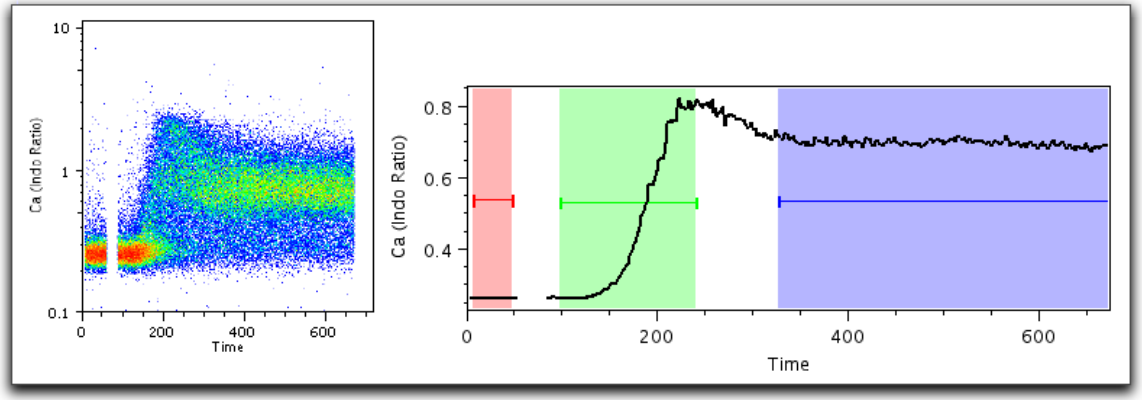
Identify each generation after division.

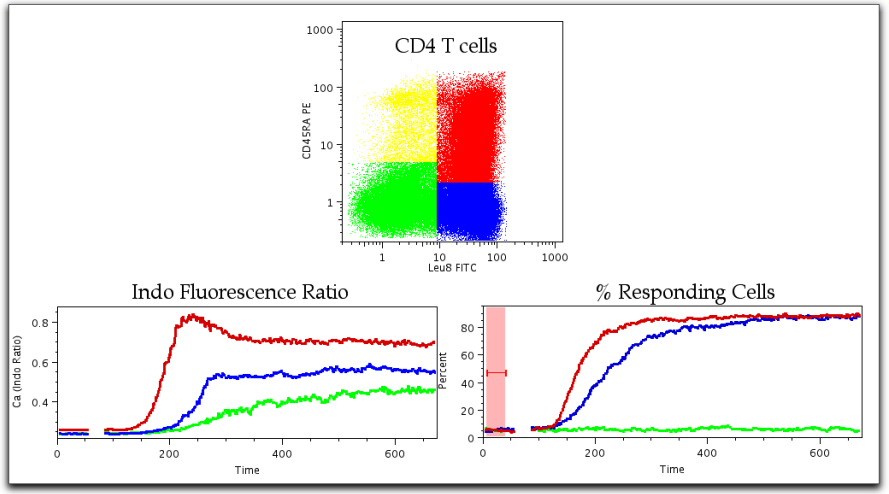




Kinetics (Calcium flux)

Calculate and display time-dependent data.





Complexity Reduction Algorithms

Downsampling

Dimension Reduction Algorithms

PCA: Prinicipal Component Analysis

LDA: Linear Discriminant Analysis

tSNE: Stochastig Neighbour Embedding

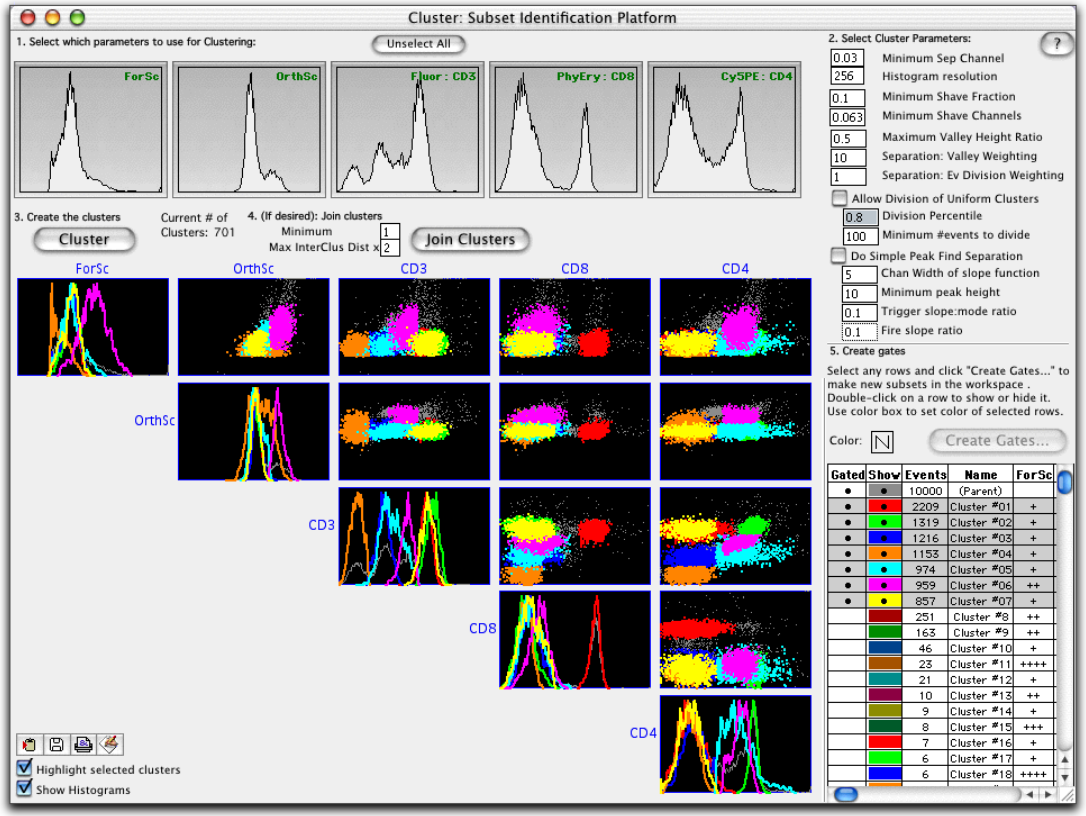
Clustering Algorithms

Probability Binning

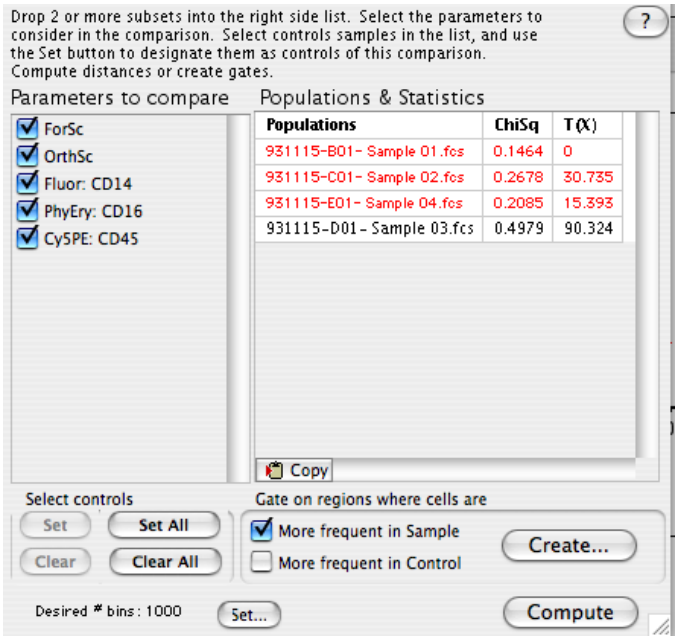
Flow Means (K-Means)

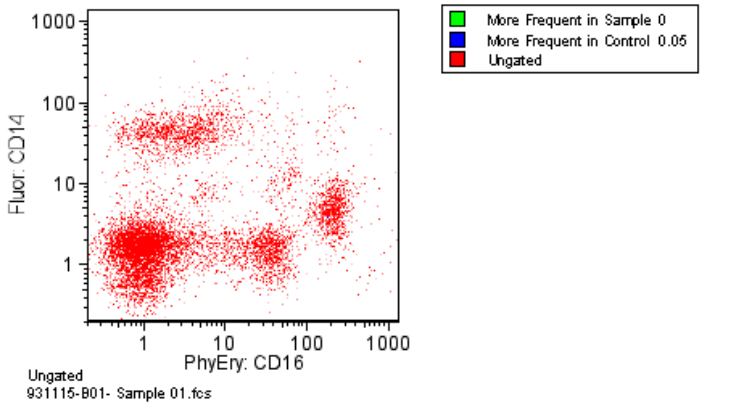
Clustering

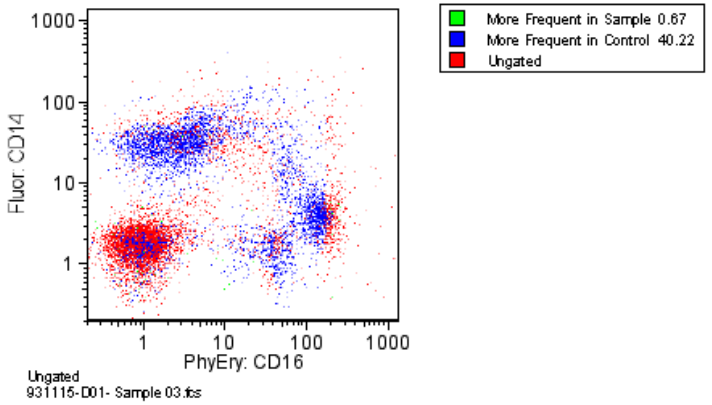
Define populations (clusters of cells) automatically.



Multisample Compare



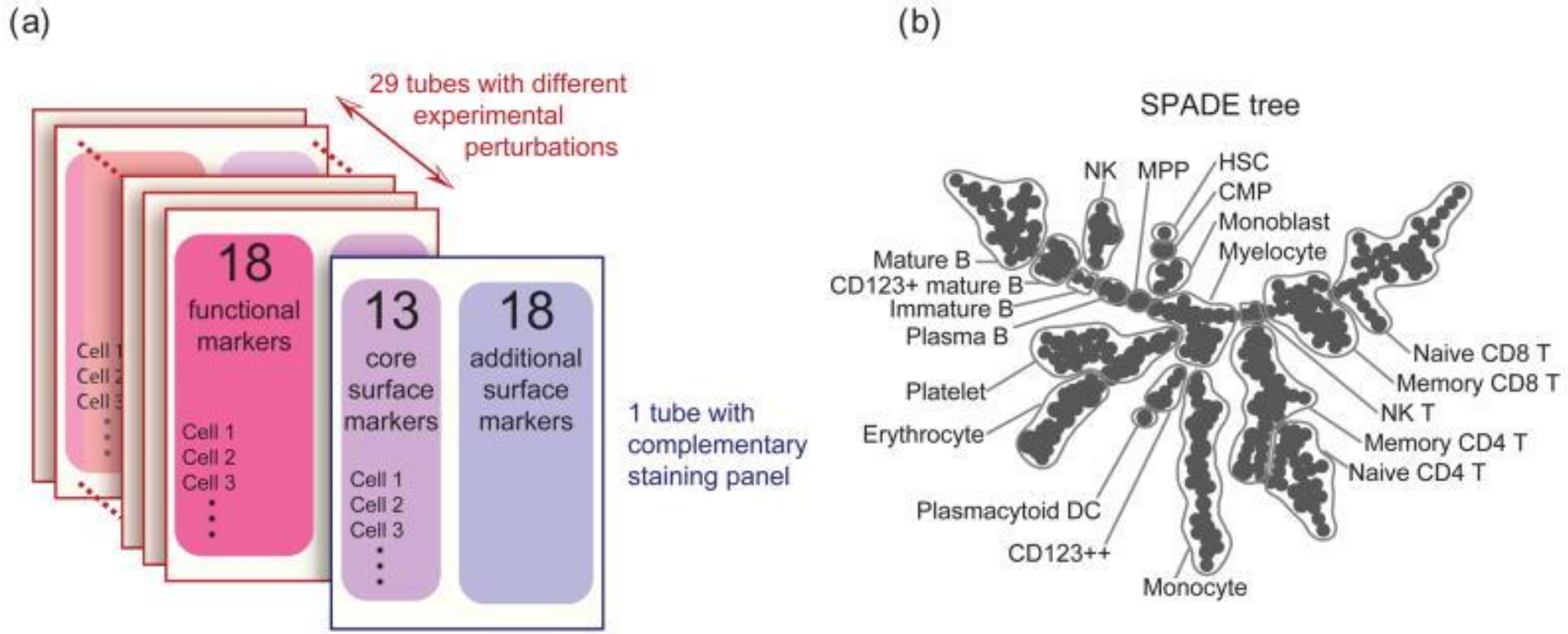




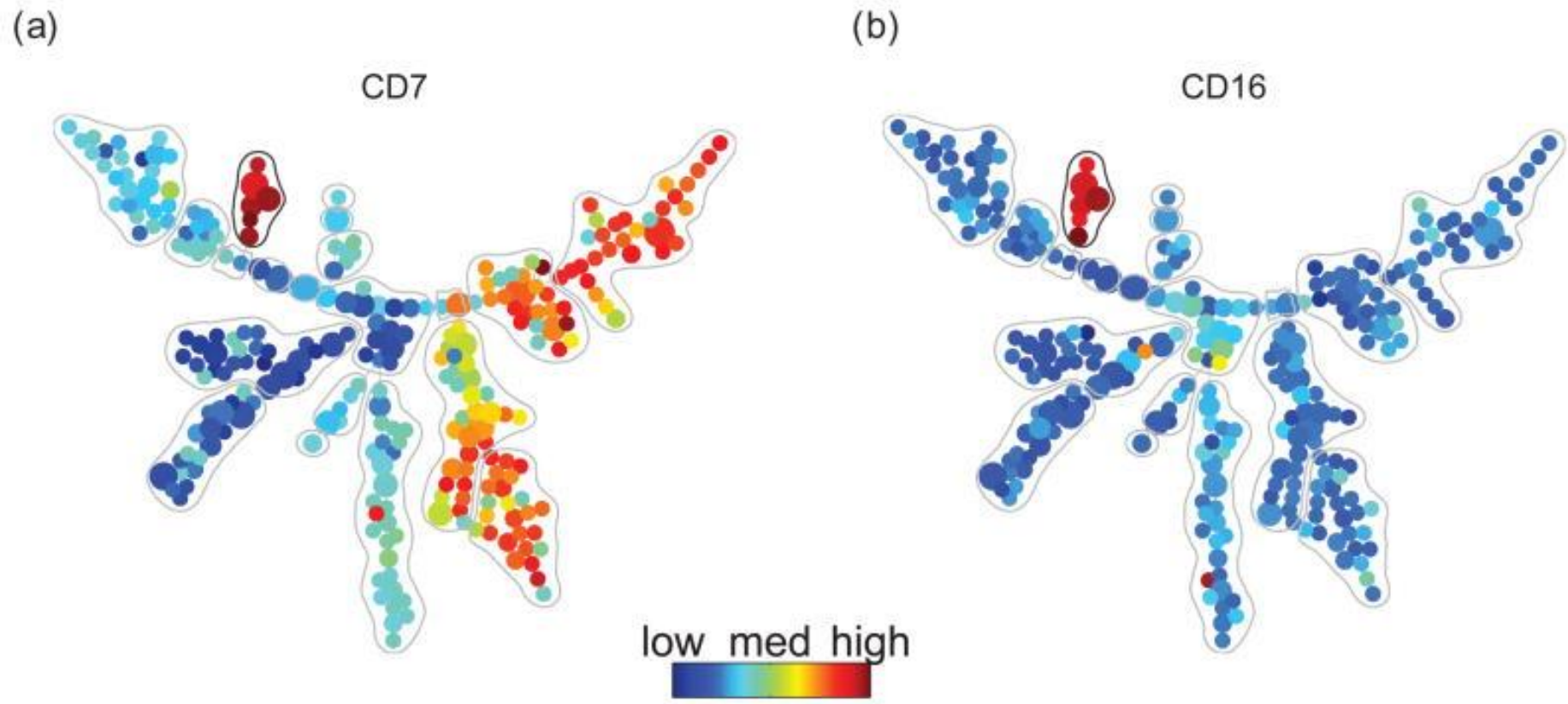
Spade

http://pengqiu.gatech.edu/software/SPADE/

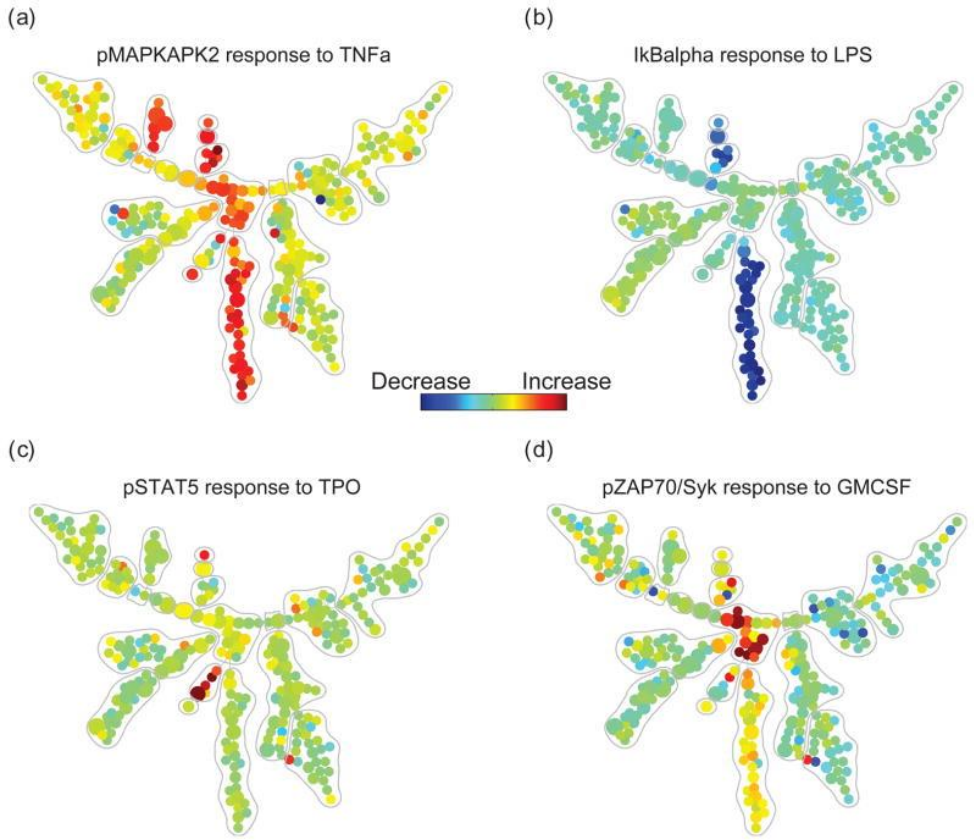
Spade


(a)
29 tubes with different experimental perturbations
18
functional markers
Cell 1
Cell 2
Cell 3
13
core surface markers
Cell 1
Cell 2
Cell 3
18
additional surface markers
1 tube with complementary staining panel
(b)
SPADE tree
NK
MPP
HSC
CMP
Monoblast
Myelocyte
Mature B
CD123+ mature B
Immature B
Plasma B
Platelet
Erythrocyte
Plasmacytoid DC
CD123++
Monocyte
Naive CD8 T
Memory CD8 T
NK T
Memory CD4 T
Naive CD4 T

Spade



Spade


(a)
pMAPKAPK2 response to TNFa
(b)
IkBalpha response to LPS
Decrease
Increase
(c)
pSTAT5 response to TPO
(d)
pZAP70/Syk response to GMCSF

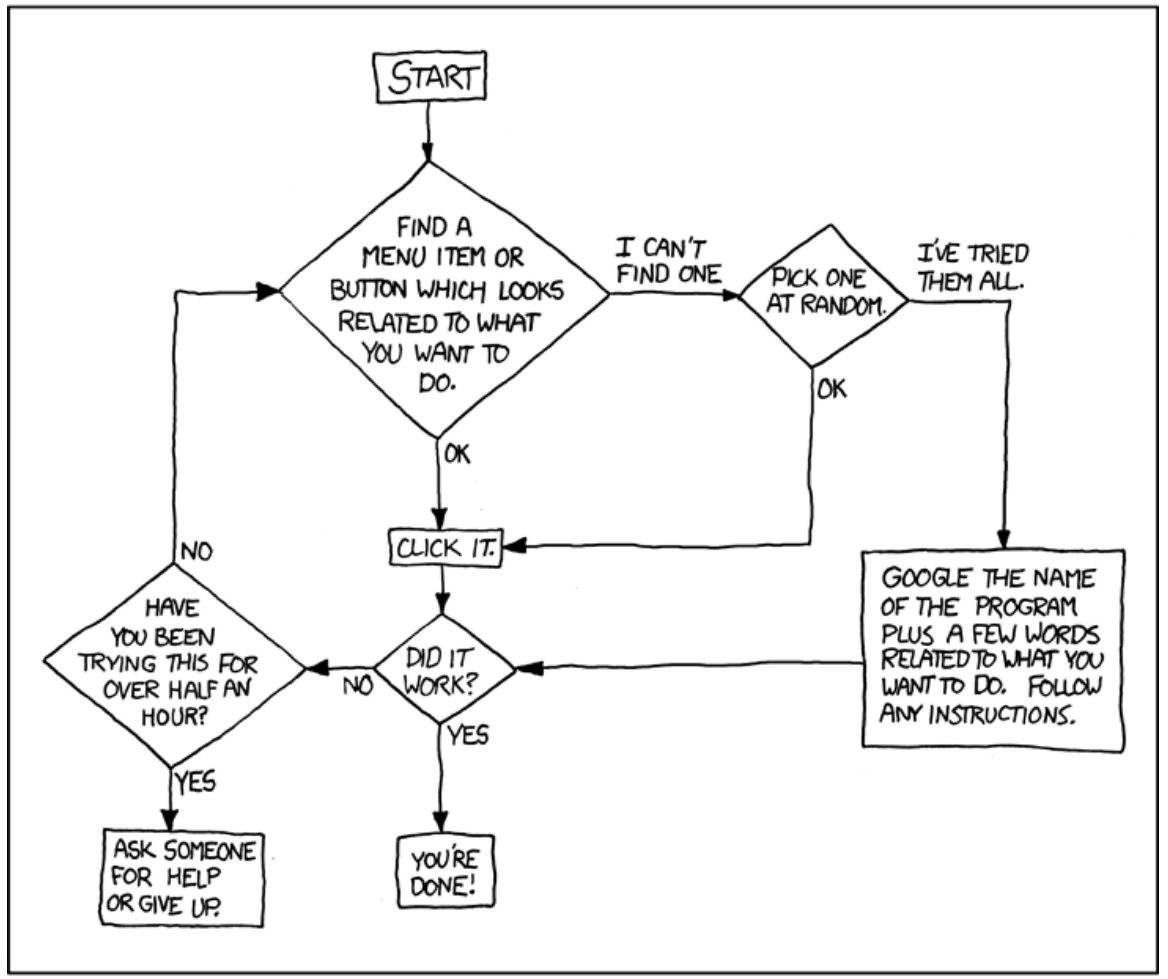
Spice

https://niaid.github.io/spice/

Data Mining & Visualization Software for Multicolor Flow Cytometry

How to become an expert:

(with any software)



Source: https://xkcd.com/627/