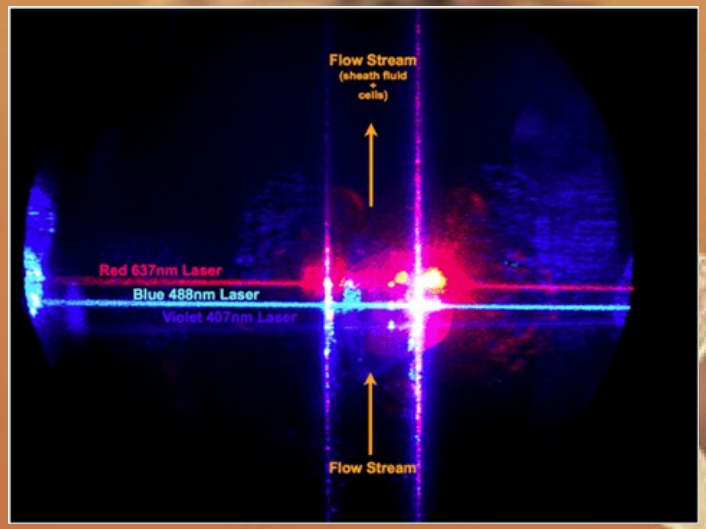
# Basics of advanced FlowCytometry

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Department of Immunology

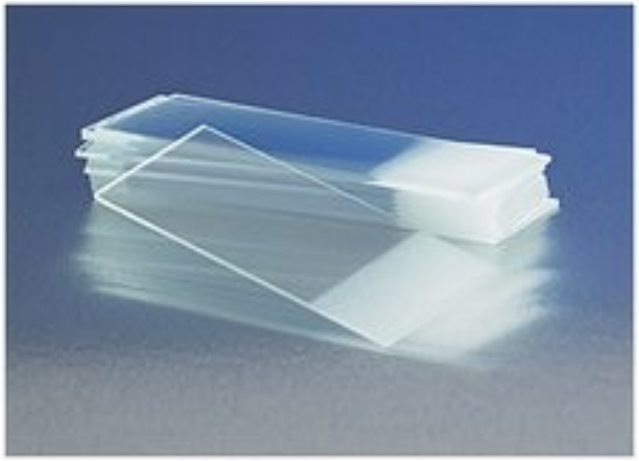
University of Pittsburgh School of Medicine
Biomedical Science Tower

Pittsburgh, PA

# Tissue disruption for single-cell suspensions

In media or staining buffer

**vs.**

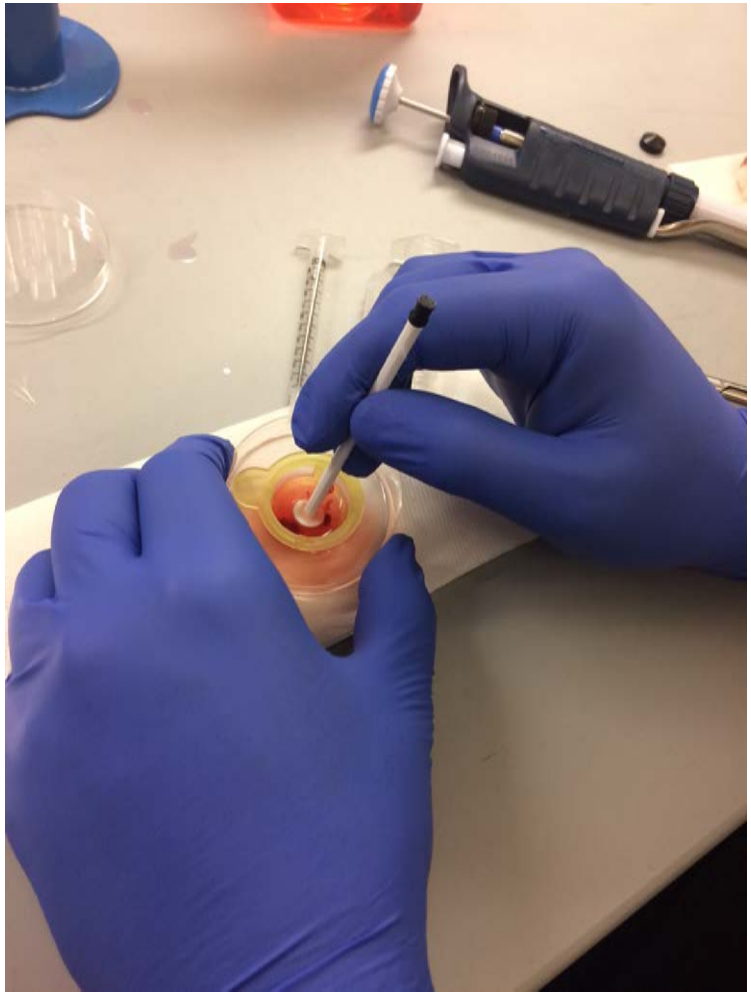



Fisher Scientific
Part no 1255310

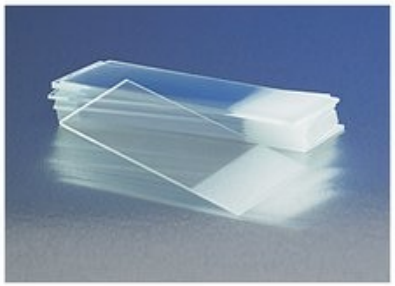
# Tissue disruption for single-cell suspensions

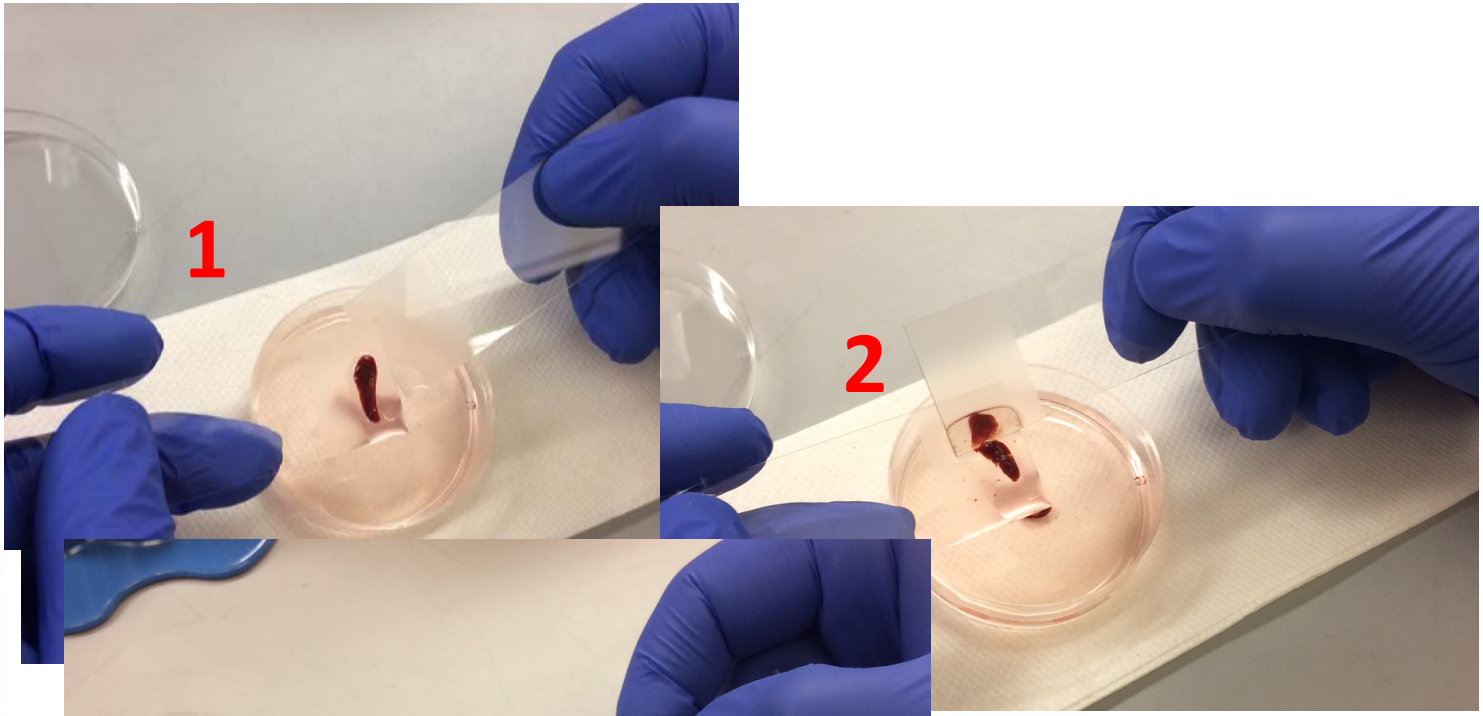
In media or

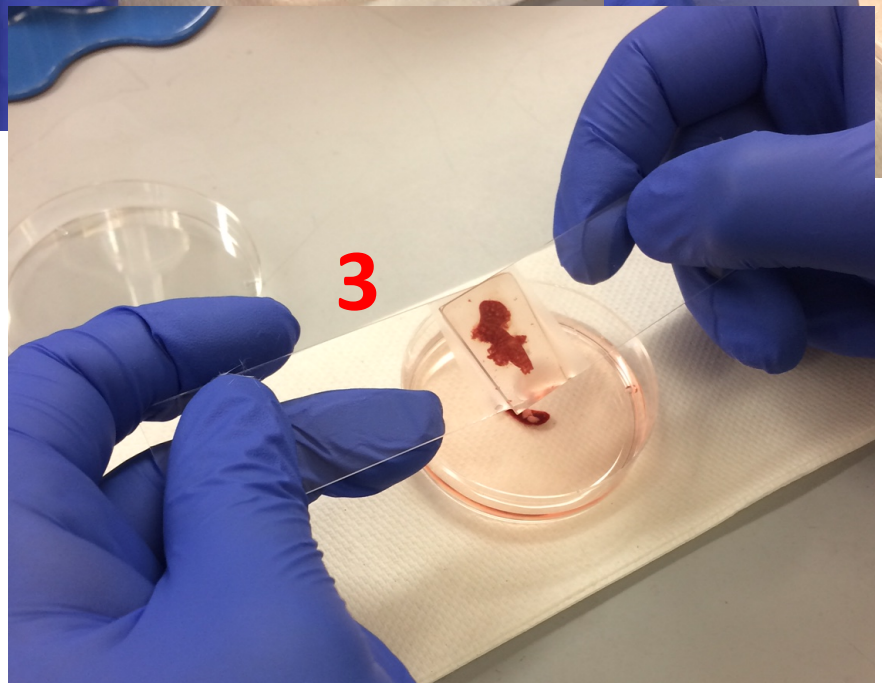
Staining buffer



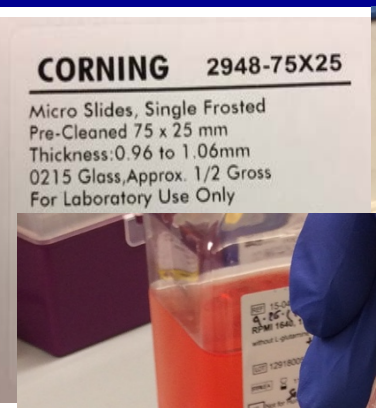
# Tissue disruption for single-cell suspensions

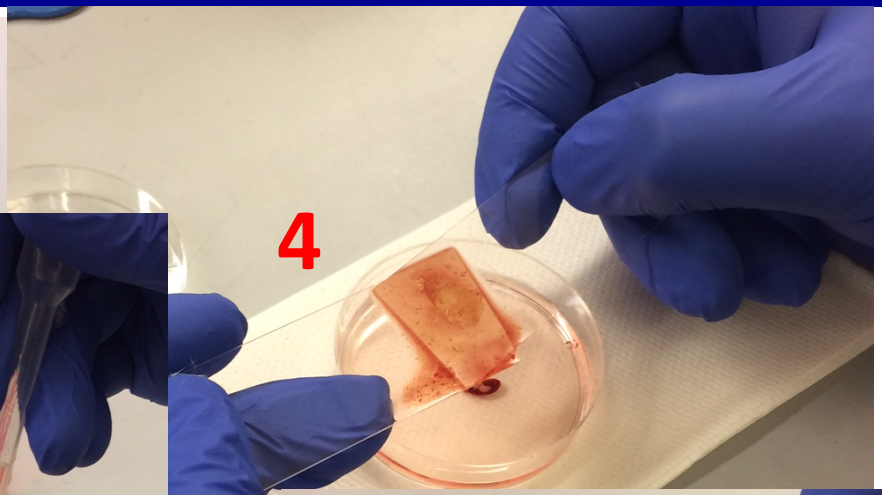



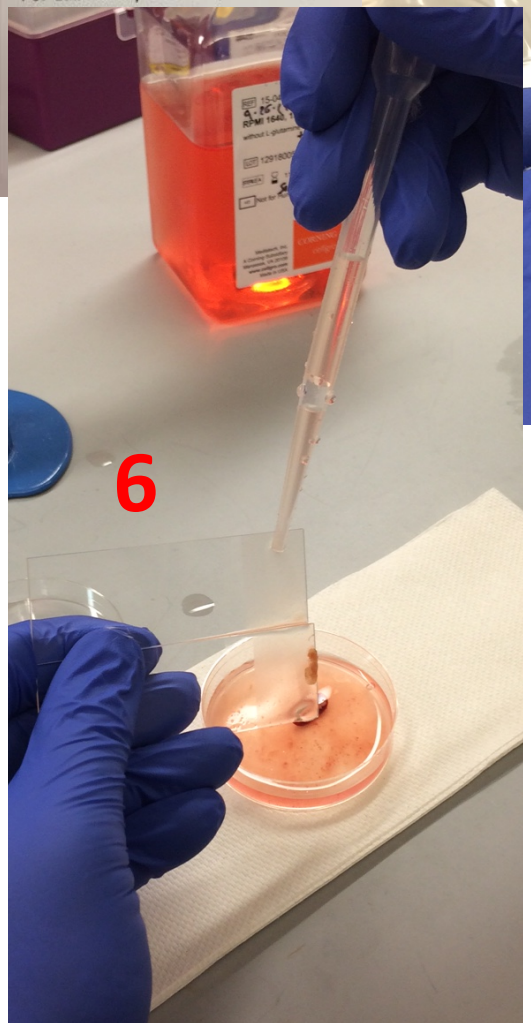


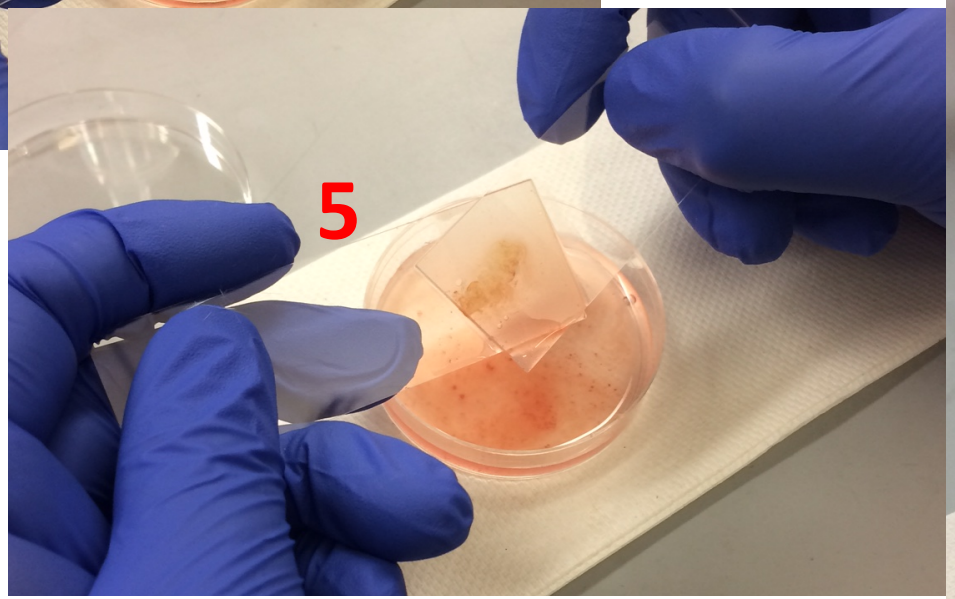



# Tissue disruption for single-cell suspensions




# Organ dissociation
# gentleMACS Octo Dissociator (Miltenyi)

**HBSS Mg+ Ca+**
**ColD (45U/ml)**
**DNaseI (80U/ml)**
**3% FCS (HI)**

Heat inactivate FCS:
30min @ 56°C


# staining

**Staining Buffer:**

PBS

3% FCS

0.02 – 0.05% $NaN_3$ (membrane turn-over; contaminations)

2mM EDTA (chelating agent; complexes $Ca^{2+}$, $Mg^{2+}$)

Always stain on ice!

Check antibodies for precipitation!

Do not use $NaN_3$ if cells are used for functional assays!

Stain $6x10^6$ lymphocytes in 50µl (=$120x10^6$/ml)

**For lymphocytes:**
**Spin 14000 rpm (= 370g) 1min per 2ml**

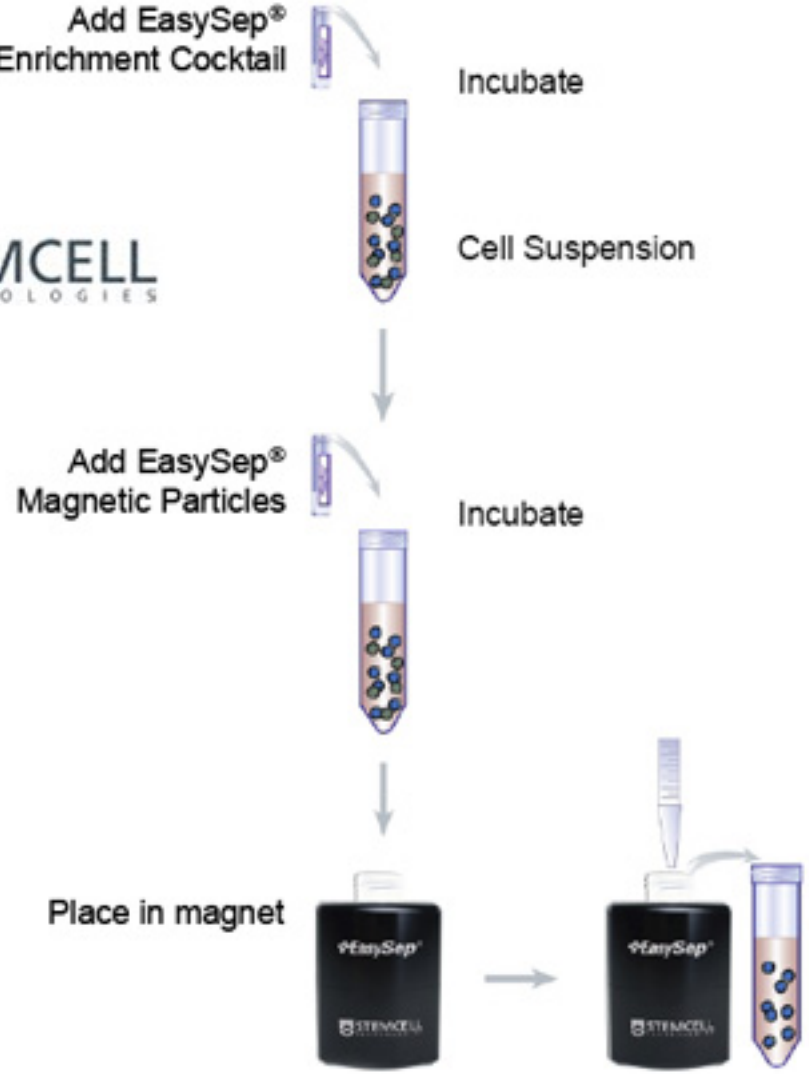
# Cell filtration


Elko Filtering co - Nylon Mesh 03-80/37
5 yards $255.1

# Enrichment of cell populations examples

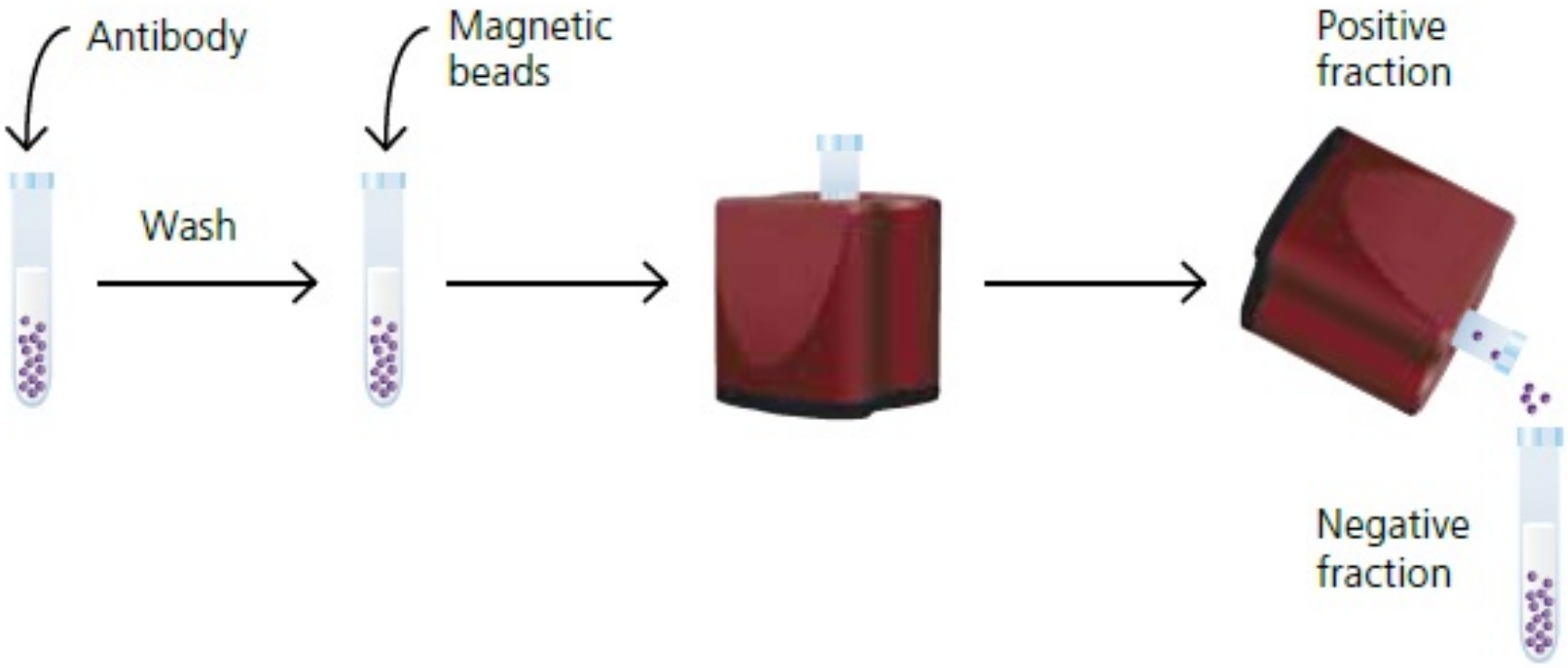
MACS
Miltenyi Biotec

STEMCELL TECHNOLOGIES




NEGATIVE SELECTION IS BETTER!!!!
…. don't touch cells of interest

# Positive / Negative enrichment




BioCell

For example: aCD19 – 1mg for $95
Biotinylate in own lab

# Un-touched enrichment of cell populations
# A cheaper way

## Cells + biotinylated ab-cocktail

+


80µl
per
$1x10^8$ cells

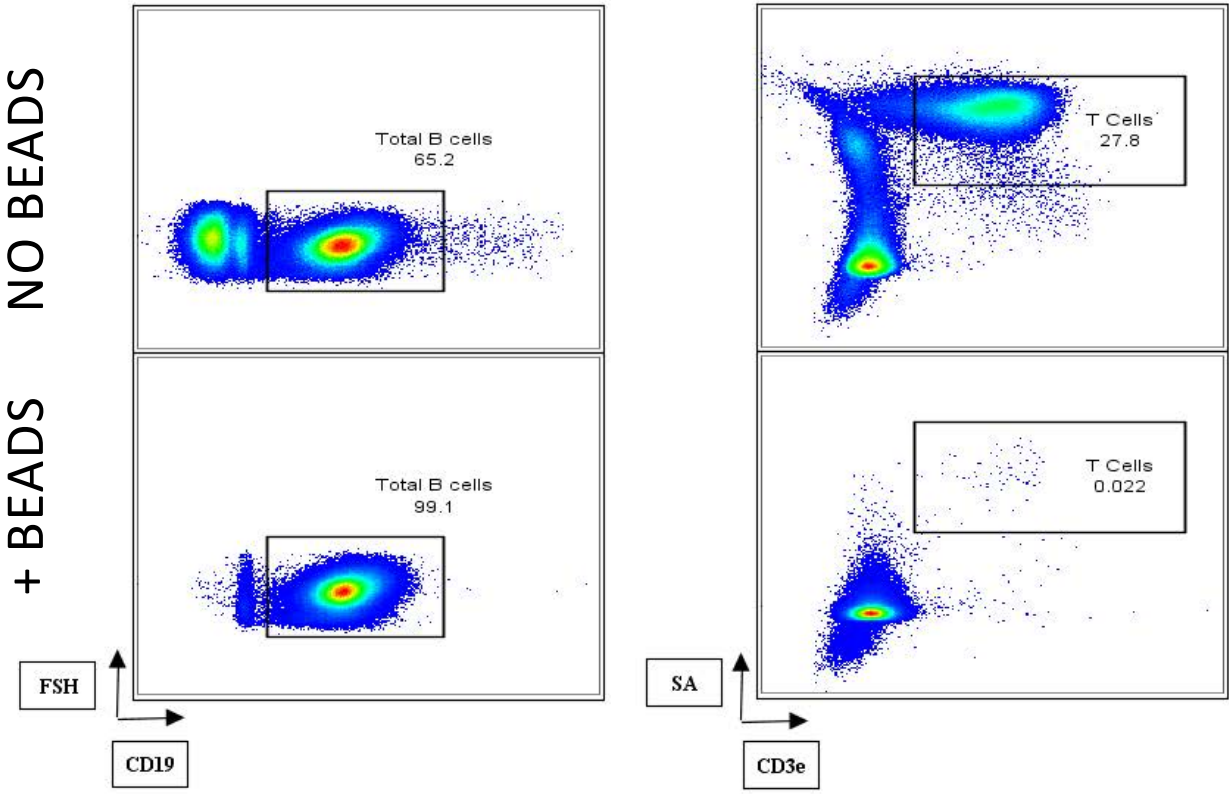
5ml = $790
65 spleens
$6.5x10^9$ cells


EasySep $1x10^9$ cells $550
Miltenyi $1x10^9$ cells $550

Beads are six times cheaper than kit

# Un-touched enrichment of cell populations

Always do a purity check – just to be sure ….



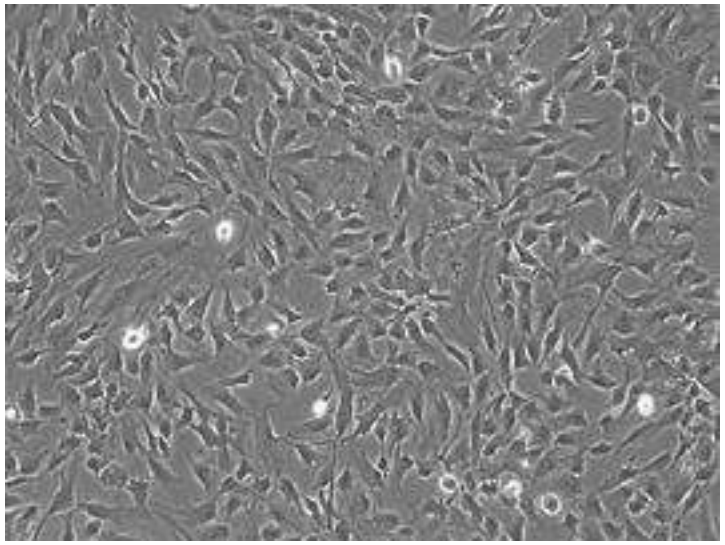

TCR-β, CD11b, CD43, CD49b, CD90.2,Ly-6G/C (Gr-1)

# Getting adherent cells ready for flow

Can't use trypsin – cuts off all surface proteins

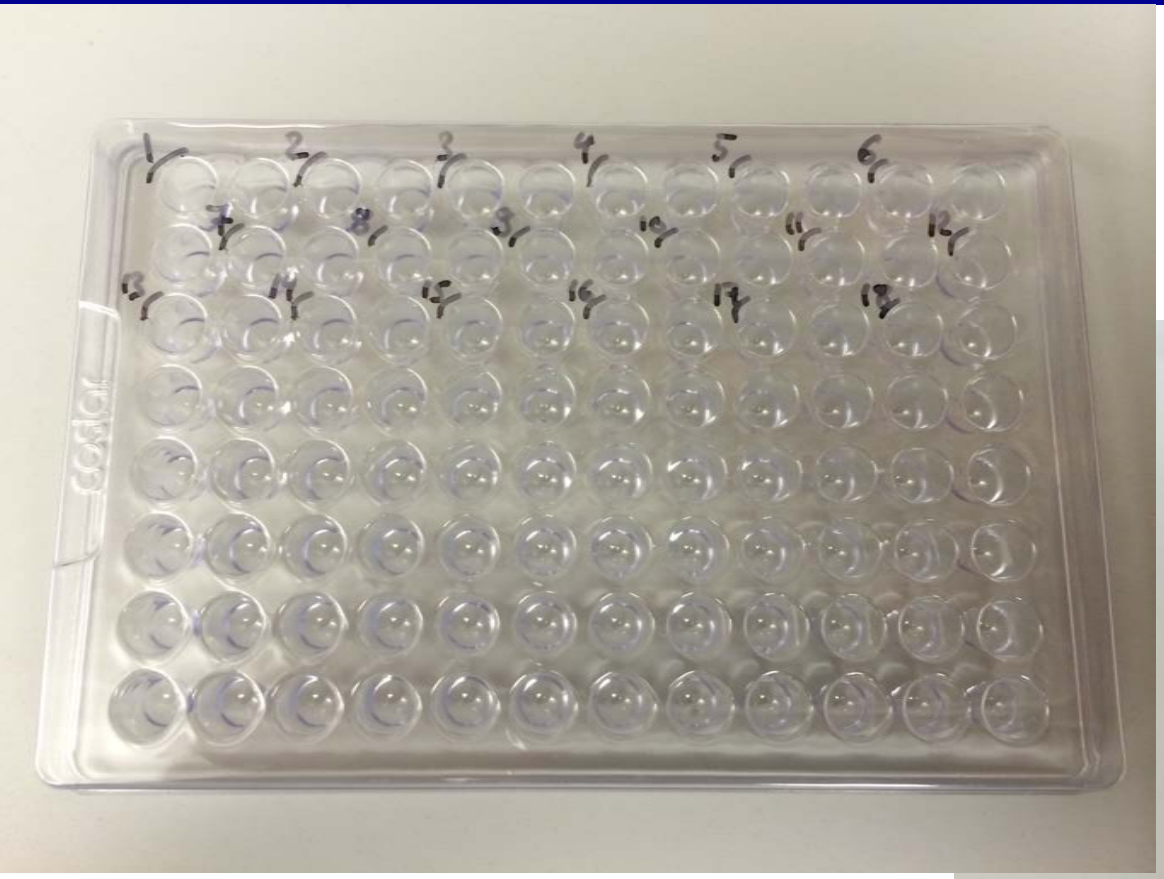
scrape

5-10mM EDTA in PBS



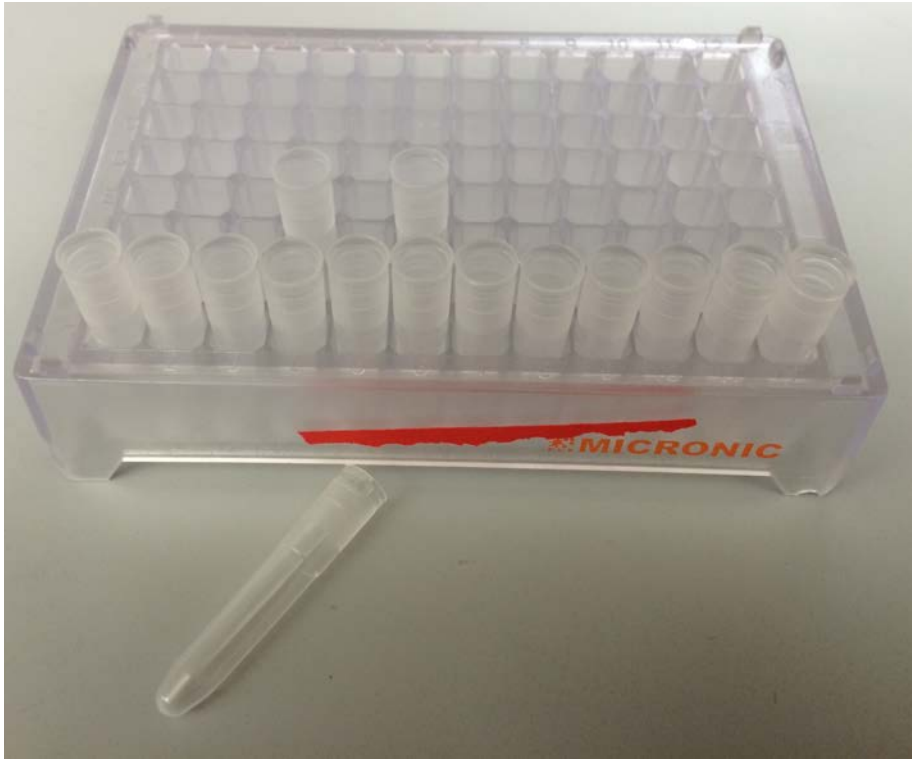
45U/ml Collagenase D (low proteinase activity) in HBSS

# Staining in plates

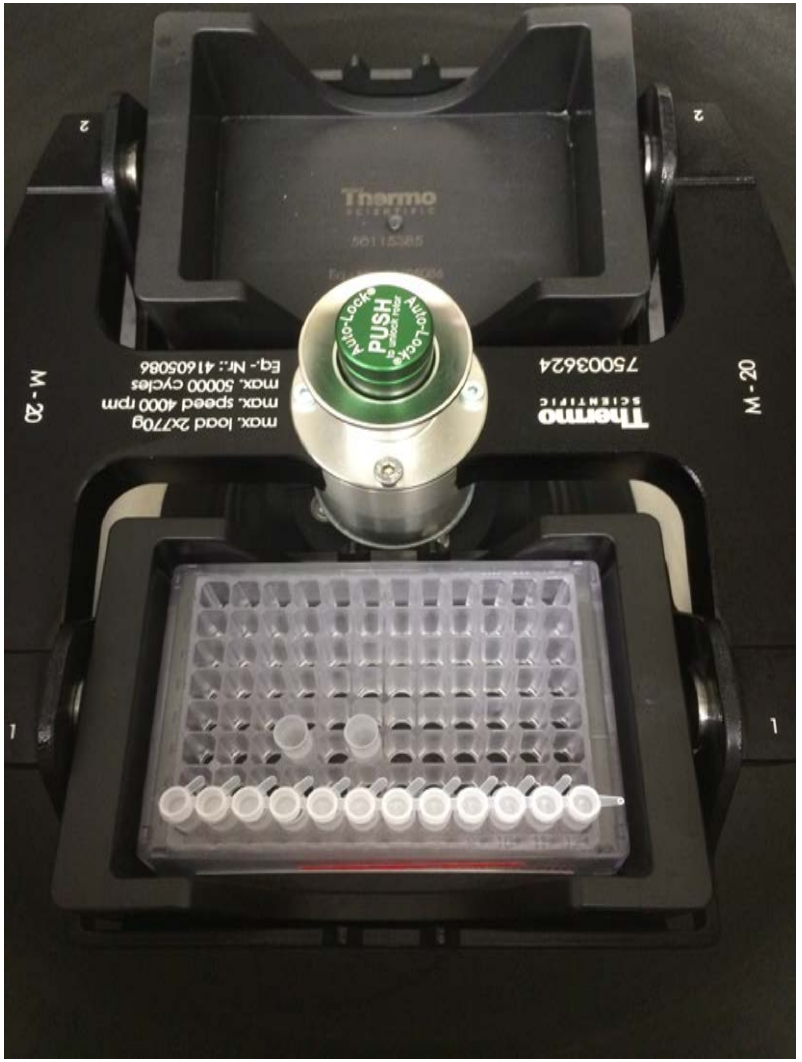


0.2ml per well,
Only use every other well

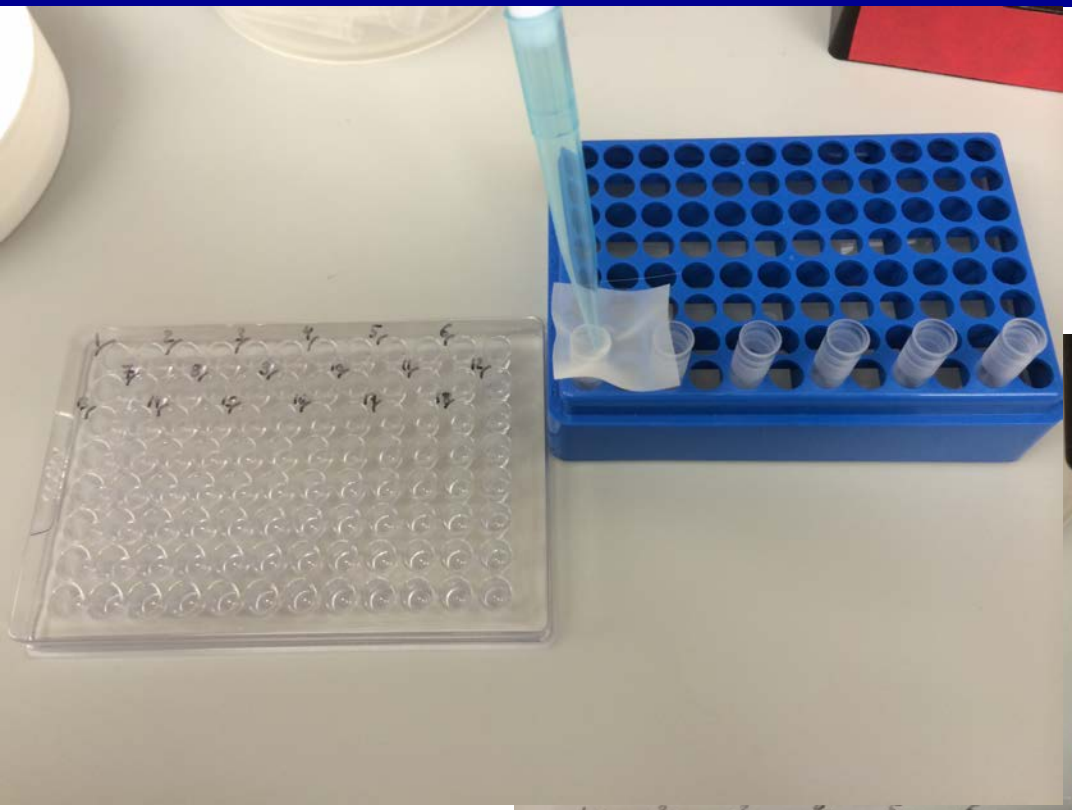
# Staining in „bullet tubes“



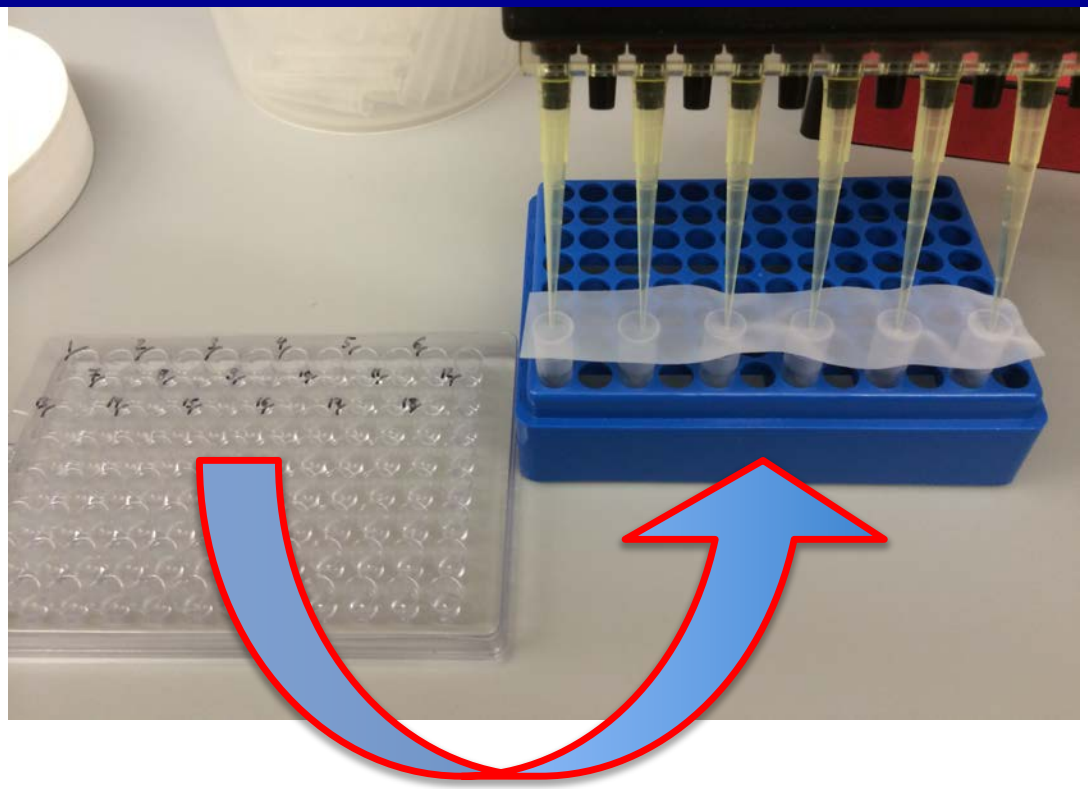

1.2ml per tube



# Staining in plates – analysis in „bullet tubes“




# Adding PI/ DAPI/ 7AAD …



Cells in 150 µl

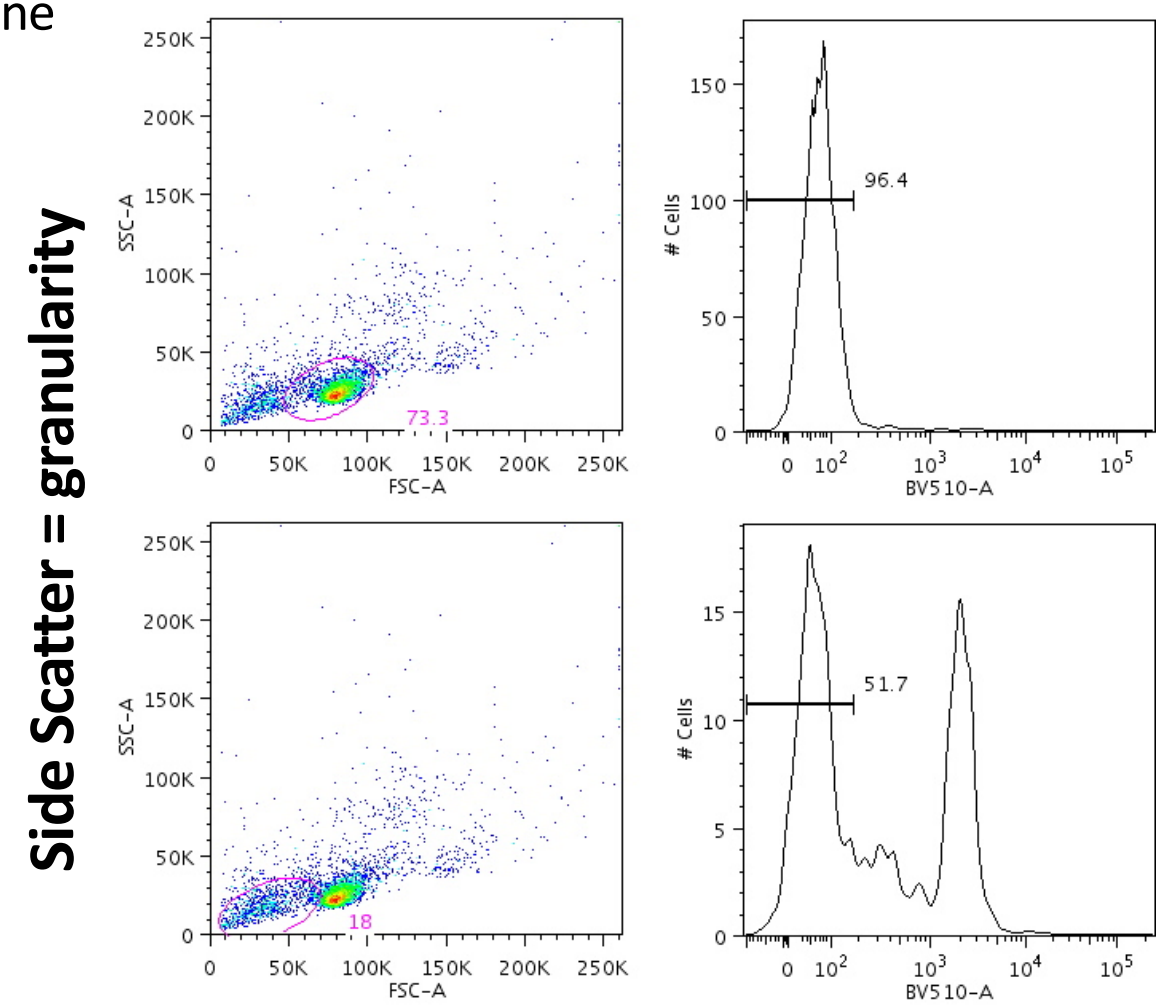
150 µl buffer with 2x PI

# live / dead staining of viable cells

If cells are alive while running on the cytometer add DNA-dye directly at the machine

PI
DAPI
7-AAD

**These dyes stain DNA** – they make it into the nucleus of dead cells and are excluded by intact cells



# Intracellular staining


This comes as 10x!!!!!

Fixation

Fixation and permeabilization For intracellular staining

# Intracellular staining

| | |
|---|---|
| Fc-block (CD16/32) +/- serum | 10min |
| ---wash--- | |
| Surface stain in SB | 30+min |
| ---wash--- | |
| 500µl CytoFix/CytoPerm | 20+min |
| ---wash in 1xPerm/Wash--- | |
| Intracellular stain in Perm/Wash | 30+min |
| ---wash in 1xPerm/Wash--- | |

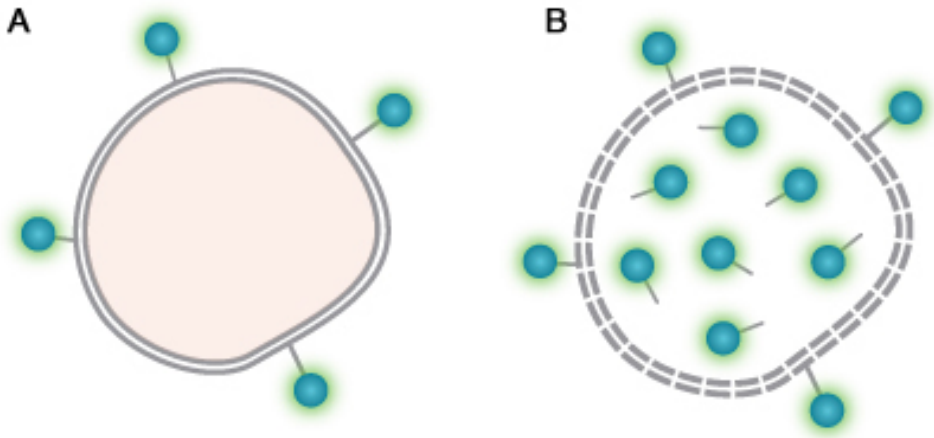
Can't use DNA dye anymore for live/dead discrimination since all nuclei are accessible – DAPI/7-AAD … can be used as DNA content measure

# live / dead staining of fixed cells

Can't use DNA dyes anymore since all nuclei are accessible.
DNA dyes can now be used for DNA content measurement - see later

Fixable Viability dyes covalently bind to **free primary amines**.
You can get them in almost all colors now (Zombie, Ghost …)
Stain with fixable viability dye prior to fixation

**A** On live cells dye stains only surface.
**B** Dead cells have compromised membrane and dye therefore also stains intracellular amines making dead cells brighter




Bio-rad.com

**These dyes essentially stain proteins and can therefore be used as size marker. Much more accurate than ForwardScatter**

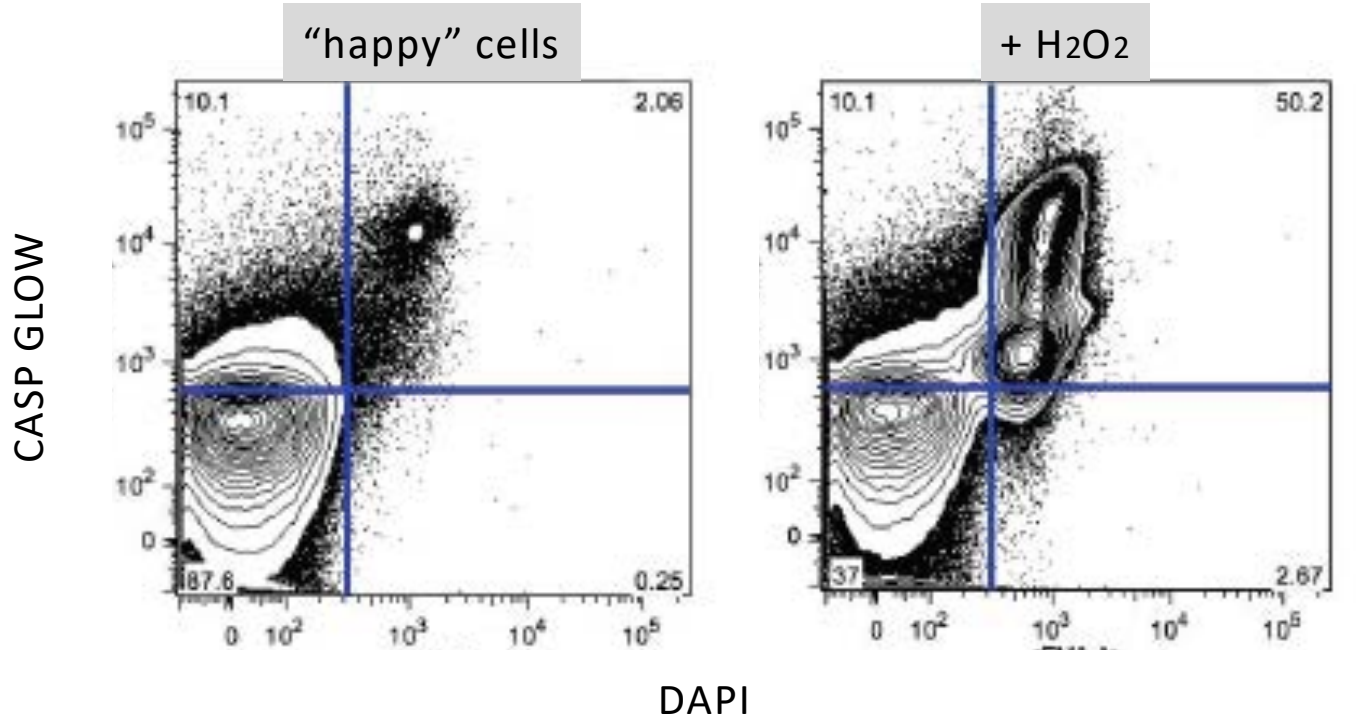
# Staining for active apoptotic cells

Apoptotic cells have active caspases

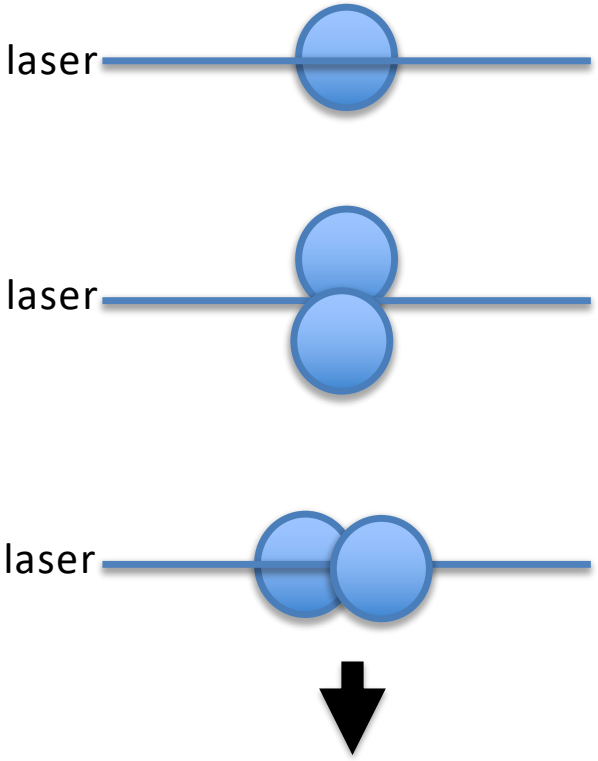
Besides expensive kits there is a great (and cheap) reagent:

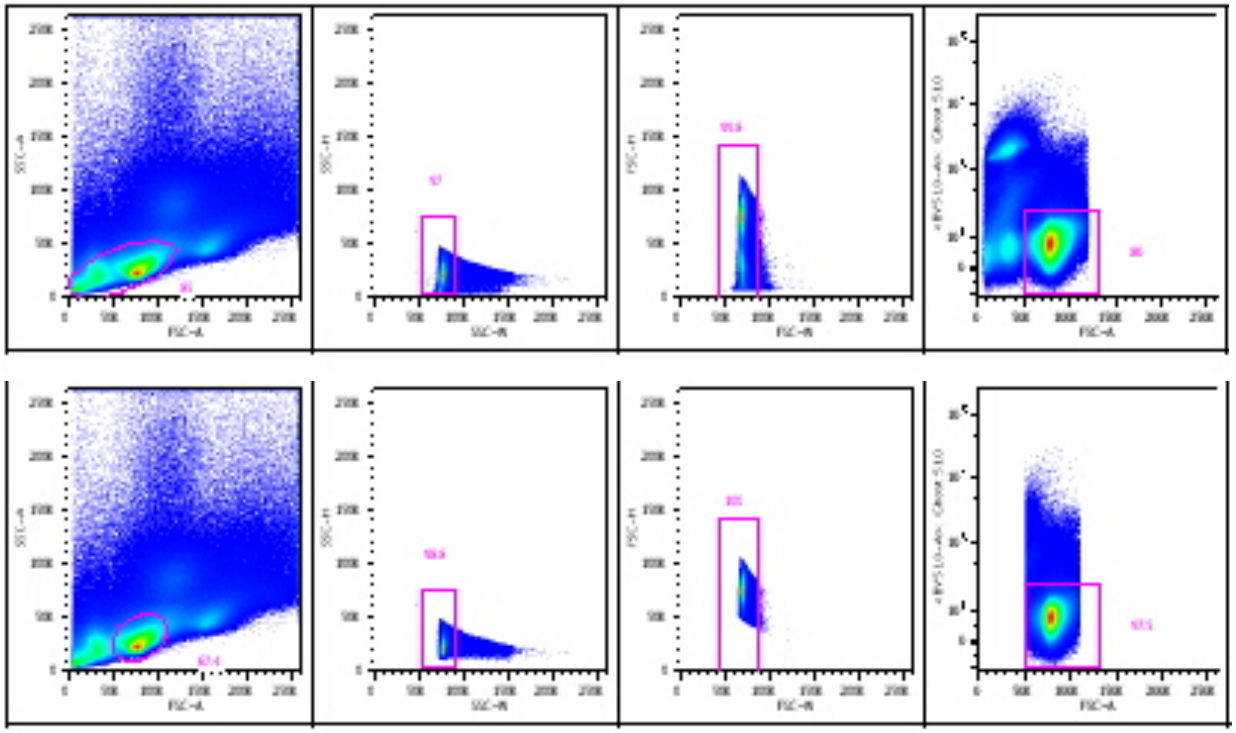
**FITC fluorescent Caspase inhibitor "CaspGlow"**
**SM Biochemicals LLC;** Cat # SMFMK020

Irreversibly binds to multiple active caspases and therefore turns apoptotic cells green upon incubation with this reagent (30-40min @ 37°C)



# singlets / doublets



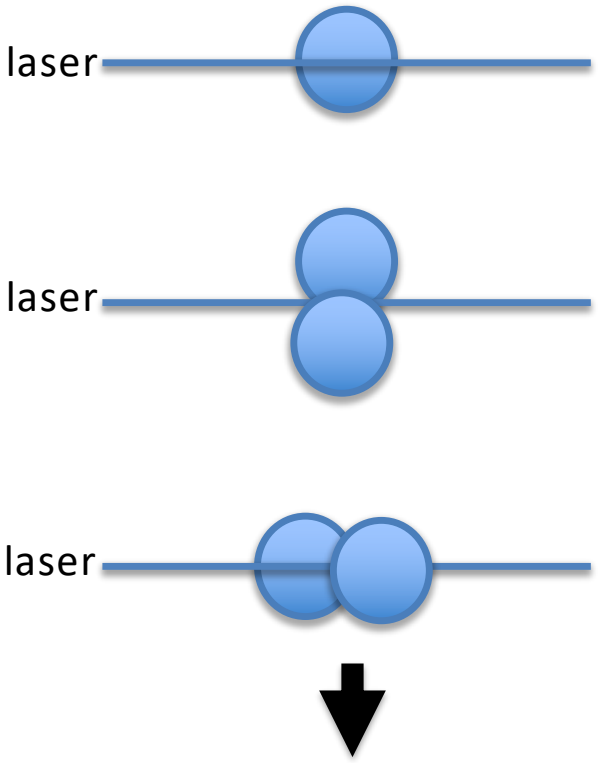



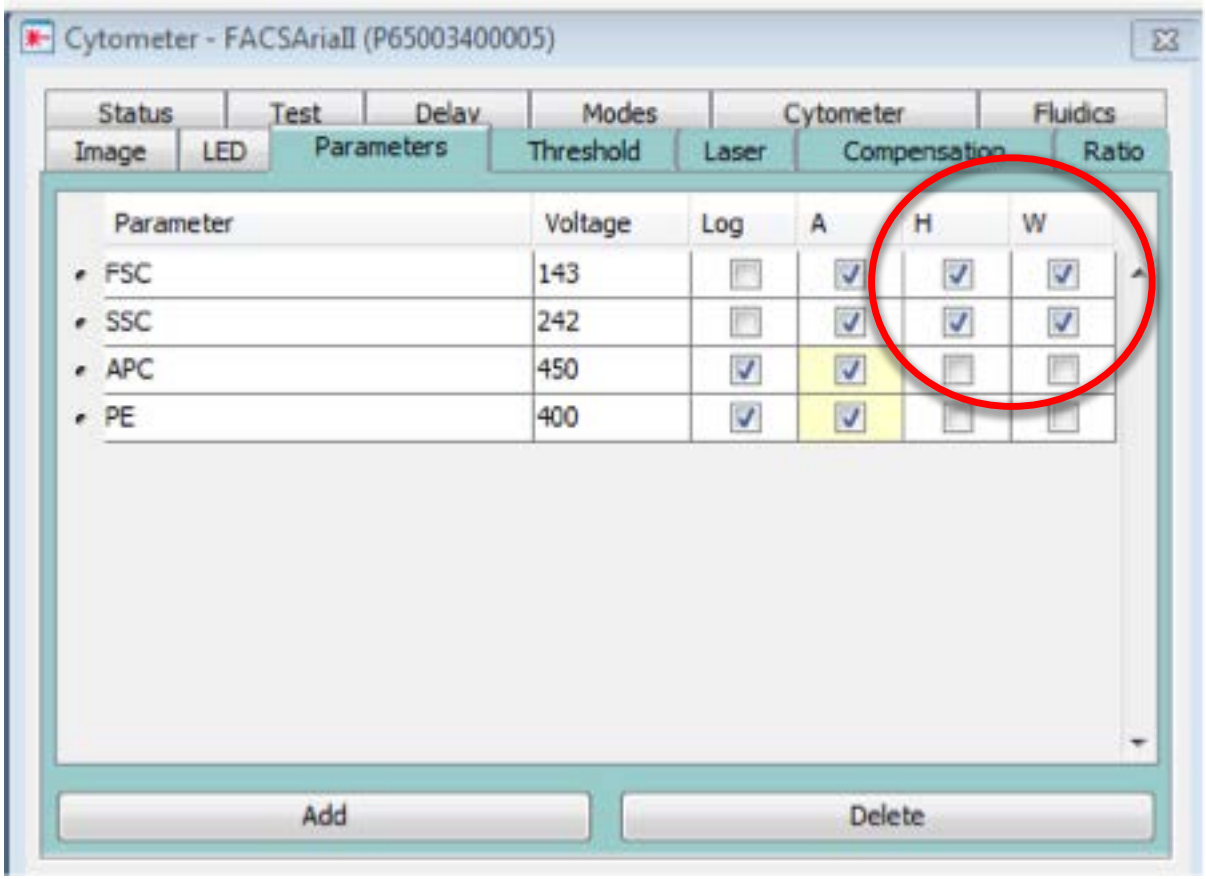
SSC-H vs SSC-W

FSC-H vs FSC-W

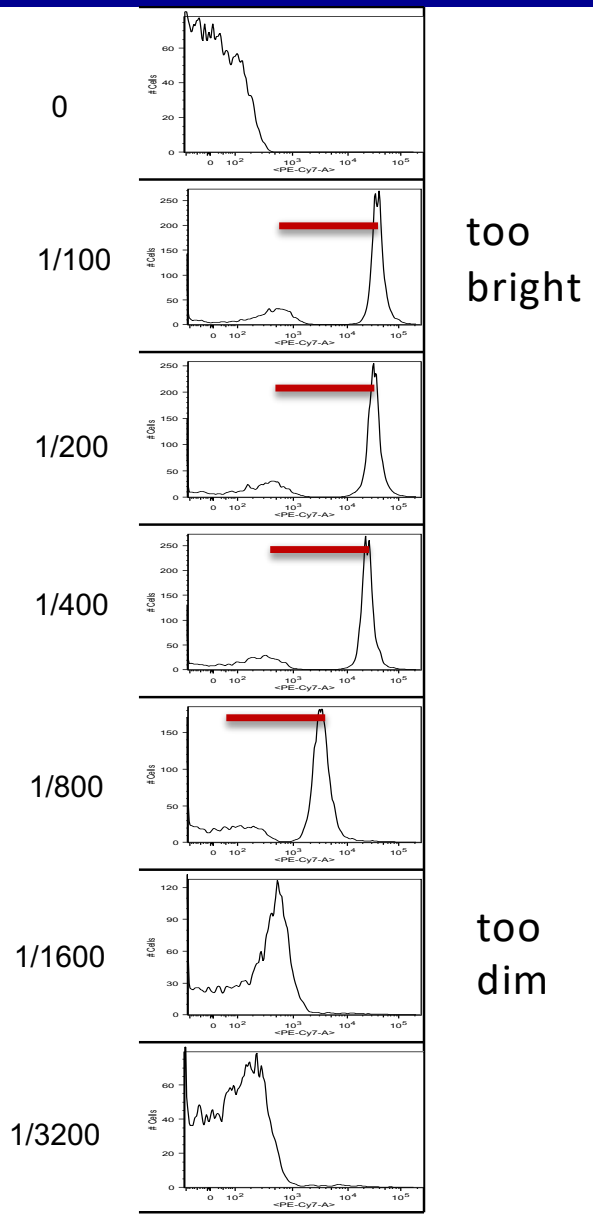
live singlet

# singlets / doublets



# Antibody titers




Always titer antibodies

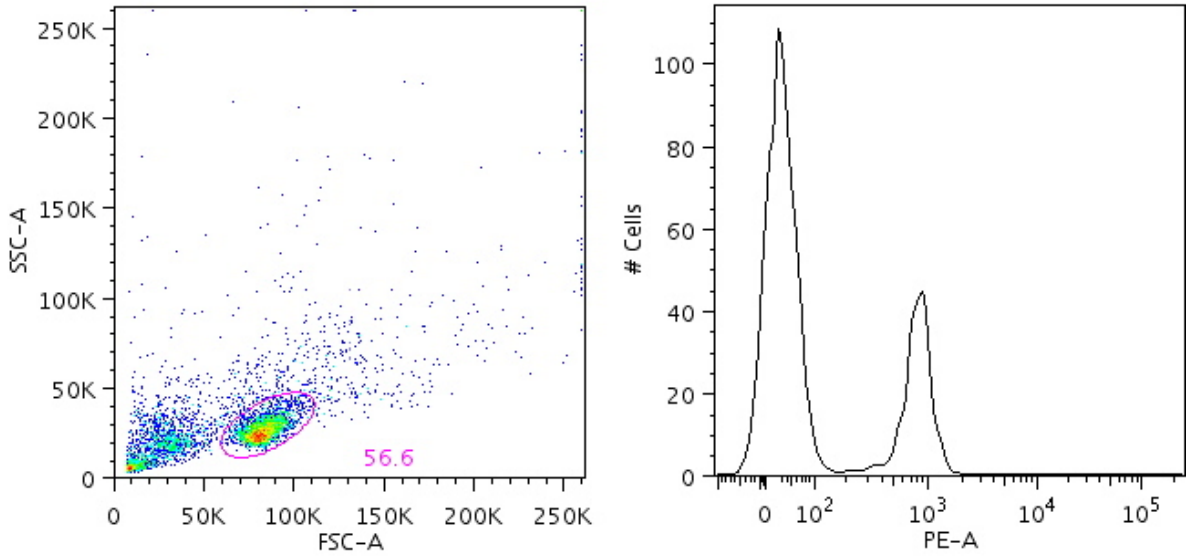
Find dilution which gives good separation with minimal background

too bright staining might lead to compensation issues

# Multicolor Flow – compensation/ single color controls

Set up voltage for each channel to define positive / negative populations

Ideally use same antibody for comp controls on the cells used in your experiment. This will result in the same brightness of all single color controls as it is in the real stain.



# Multicolor Flow – compensation/ single color controls

**3 golden rules:**

Controls need to be at least as bright as any sample will apply the compensation to.

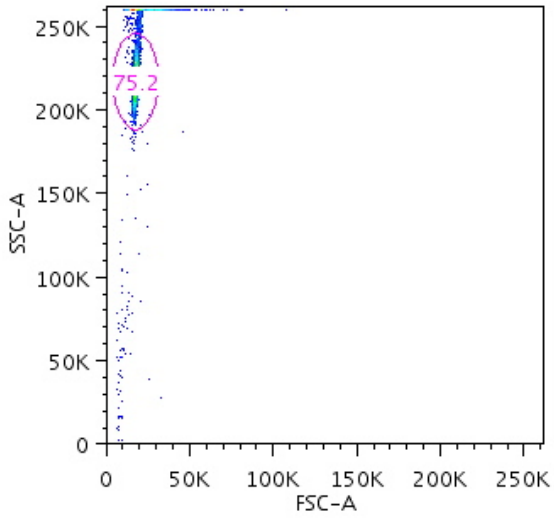
Background fluorescence should be the same for the positive and negative control populations for any given parameter.

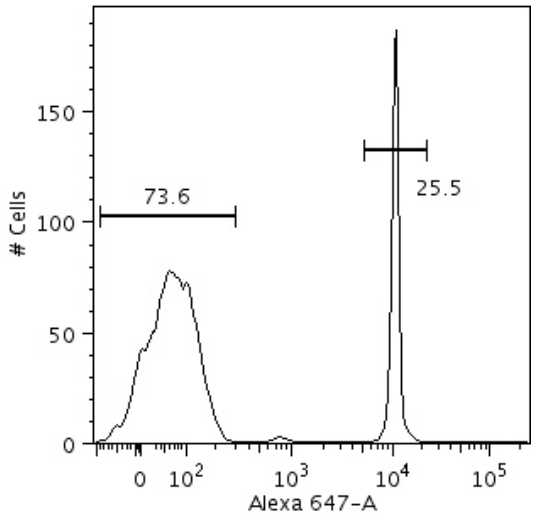
Your compensation color must be matched to your experimental color

# Multicolor Flow – compensation/ single color controls

If target population is too small better use different antibody or beads:

Mix 50:50 unconjugated beads (white; this gives the negative peak) with anti-rat/hamster (green) or anti-mouse (blue) and incubate with antibody pre-dilution in desired color

# Multicolor Flow – Check configuration of cytometer

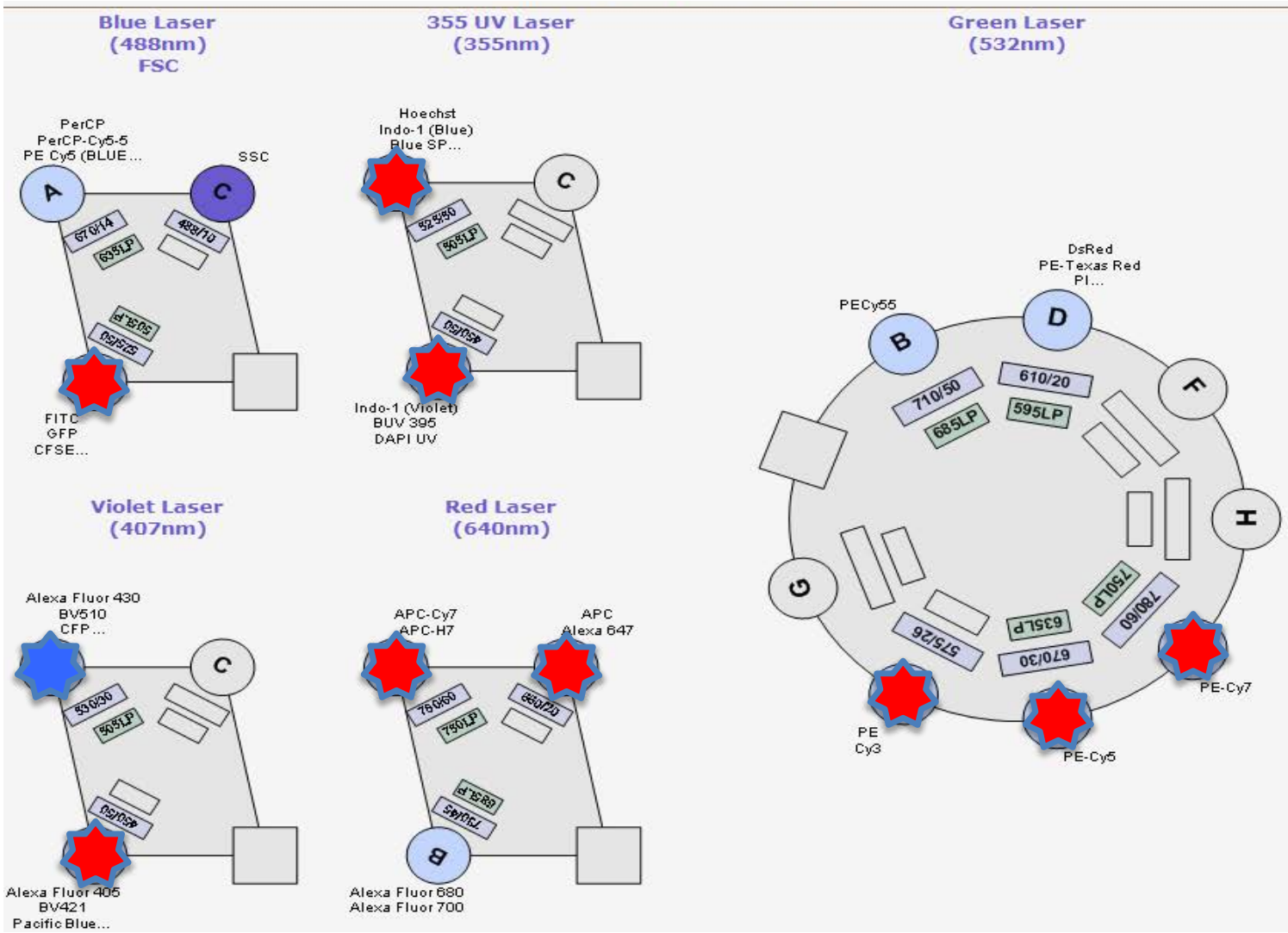
## IMMUN LSR II BST E1005

The LSR II is equipped with 5 lasers running on Diva 8/Windows 7. Lasers: 355 20mW Trigon, 405 50mW Trigon, 488 20mW Trigon, 532 150mW Octagon, 640 100mW Trigon. For configuration, click here. For Fluorofinder panel design, click here.

| DETECTOR NAME | LP MIRROR | BP FILTER | FLUOROCHROMES |
|---|---|---|---|
| 488 Laser A | 635 | 695/40 | PerCP/PerCP-Cy5.5 |
| 488 Laser B | 505 | 525/50 | FITC, GFP |
| 488 Laser C | --- | 488/10 | SSC |
| 532 Laser A | 705 | 780/60 | PE-Cy7 |
| 532 Laser B | 685 | 710/50 | PE-CS.5 |
| 532 Laser C | 635 | 670/30 | PE-Cy5 |
| 532 Laser D | 595 | 610/20 | PETxRED/PI |
| 532 Laser E | --- | 575/20 | PE |
| 640 Laser A | 755 | 780/60 | APC-Cy7 |
| 640 Laser B | 700 | 720/20 | Alexa 700 |
| 640 Laser C | --- | 660/20 | APC |
| 405 Laser A | 595 | 610/20 | BV605 |
| 405 Laser B | 505 | 525/50 | V500, BV510, Alexa 430 |
| 405 Laser C | --- | 450/50 | Pacific Blue, Alexa 405, BV421,V450 |
| 355 Laser A | 690 | 740/35 | BUV 737 |
| | 505 | 530/30 | Indo 1 |
| | | 670LP | Red Side pop. |
| 355 Laser B | --- | 379/28 | BUV 395 |
| | | 405/20 | Indo-1 High |
| | | 450/50 | DAPI, Alexa 350, Indo Violet |

# 10 color flow example

| |
|---|
| FVS510 |
| CD38-BUV395 |
| CD138-APC |
| CD27-BV421 |
| CD95-FITC |
| CD10-PE-Cy5 |
| IgG-biotin+SA-BUV737 |
| CD19-**APC-Vio770** |
| IgM-PE |
| IgD-PE-Cy7 |

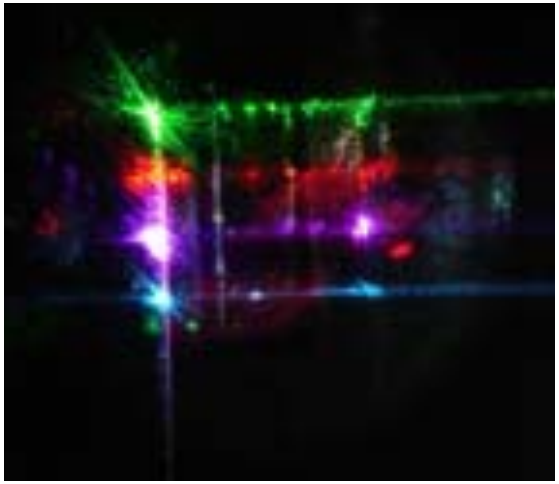


# Cytometer setup

CHANGING FILTERS – bring it back and change to default configuration. If weird signal ask flowCore to check filters

USE CST SETTINGS or NOT????

**YES – USE CST SETTINGS**



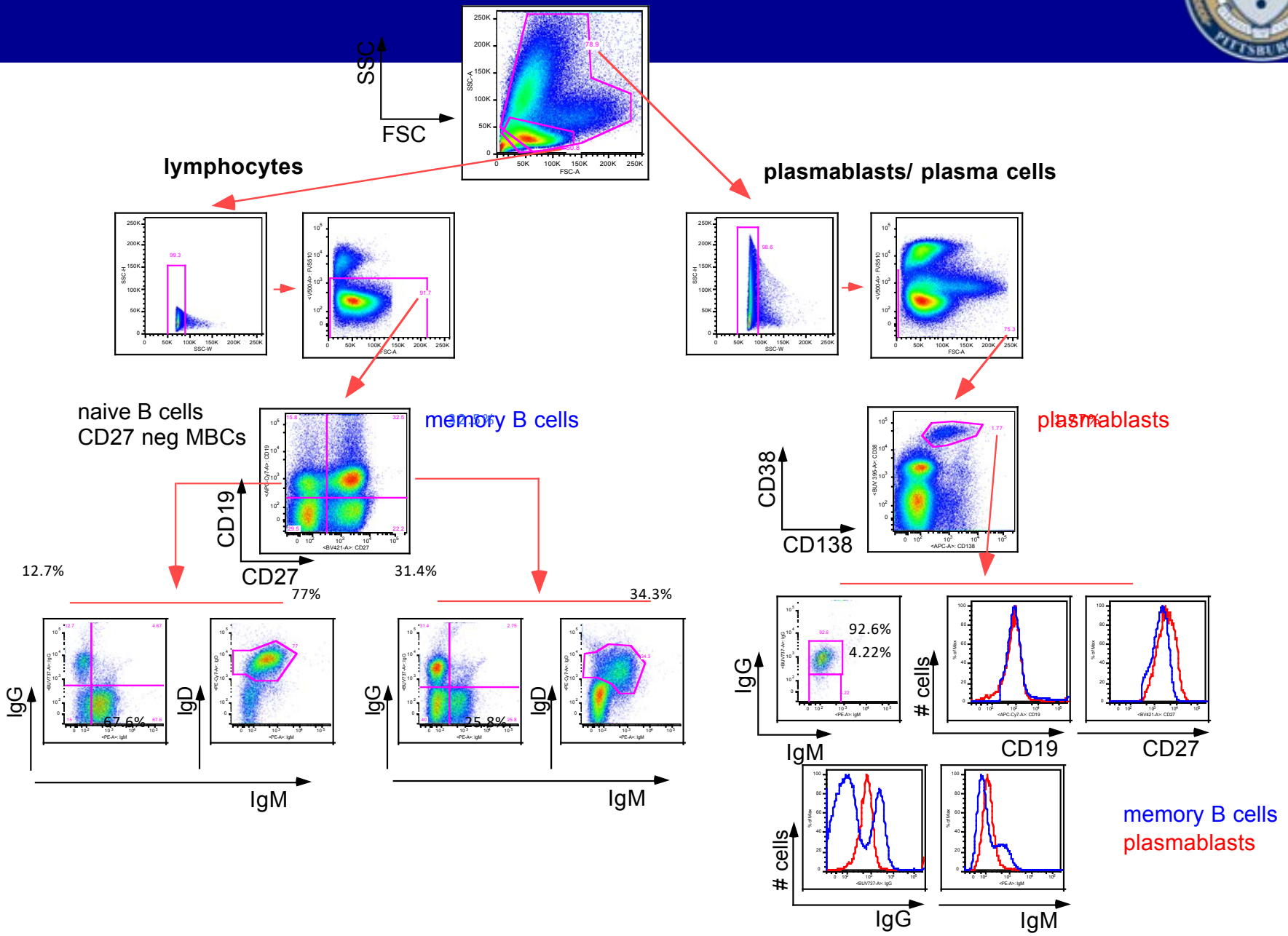
Voltage and laser delay – green laser has largest delay and therefore is impacted most.

Watch cells for shifting

Blue laser is always zero (=reference)

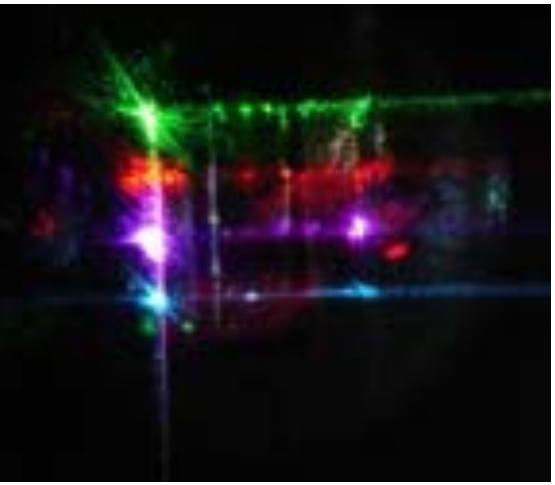
Use low FCS concentration (below 0.5%) during sample run to minimize background – especially for fixed samples

# 10 color flow - - example (LSRII)



# Compensation

Wikipedia:
In cytometry, compensation is a mathematical correction of a signal overlap between the channels of the emission spectra of different fluorochromes.



**An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers**

BD Biosciences, San Jose, CA

https://www.bdbiosciences.com/documents/Compensation_Multicolor_TechBulletin.pdf

# Compensation

Spillover is due to the physical overlap among the emission spectra of certain commonly used fluorochromes.
Spillover occurs whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signal from another fluorochrome (Figure 1).

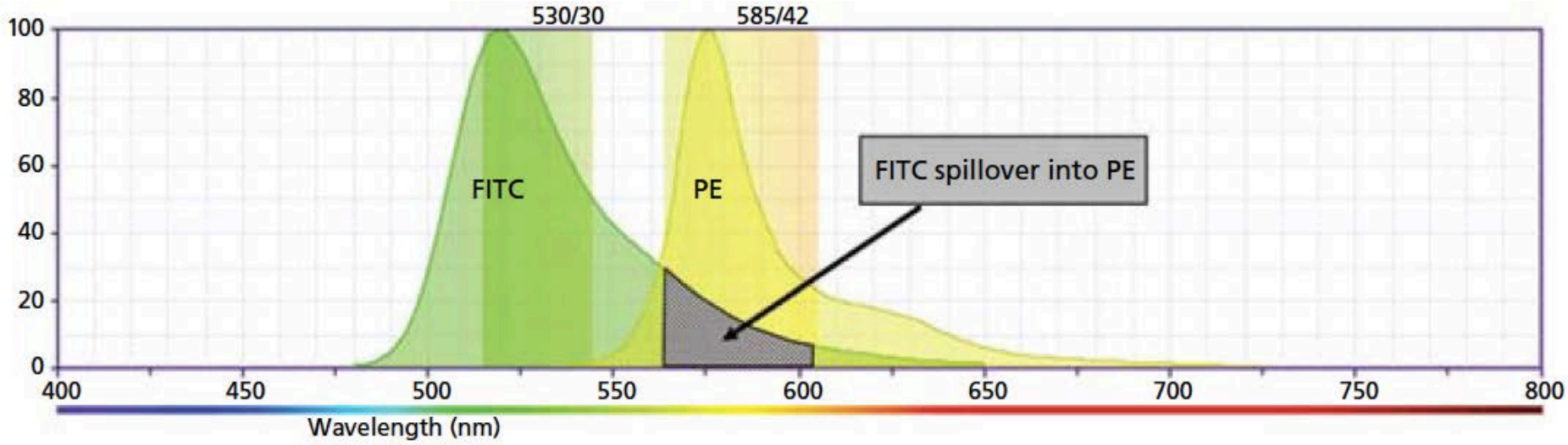
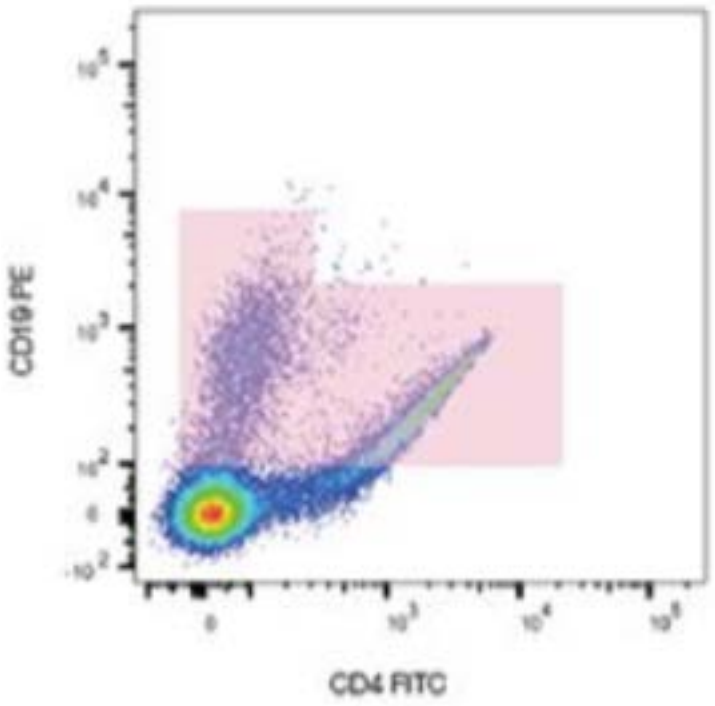



**Figure 1.** Example of FITC spillover into the PE channel.

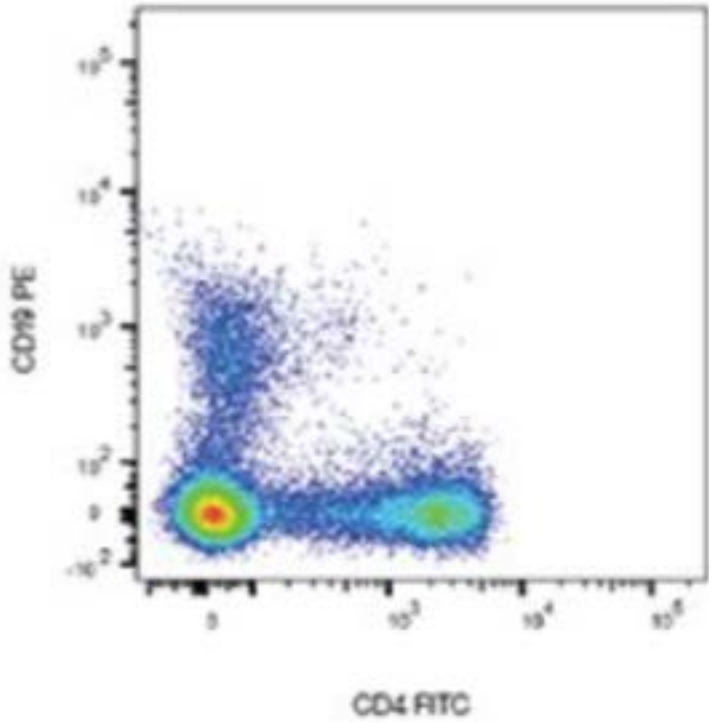
You can see from emission spectra of FITC that part of the signal is also detected by the 585/43 bandpass filter which is supposed to pick up PE signal

# Compensation




Uncompensated

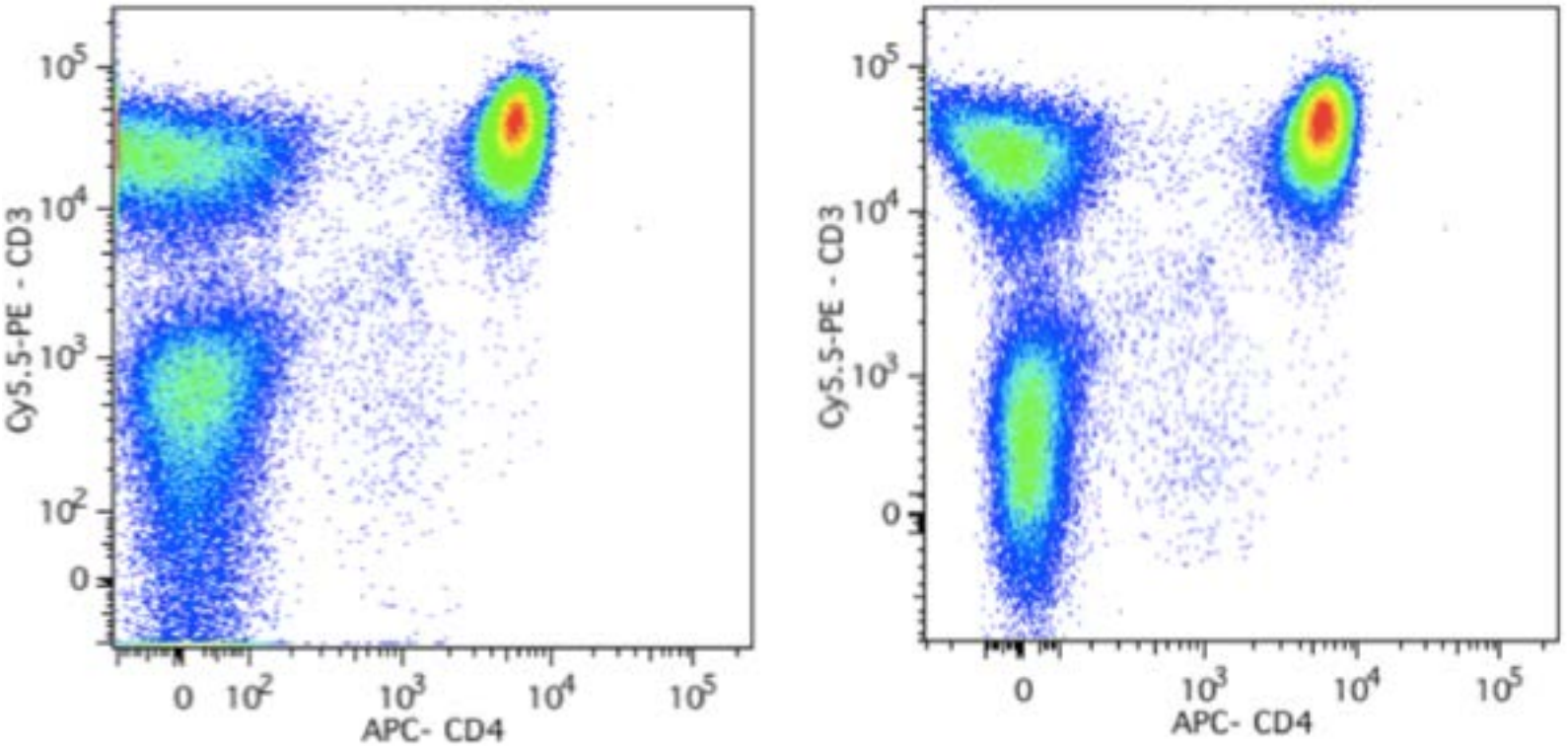
FITC spills into PE




Compensated
(median of FITC negative and FITC positive line up)

https://expertcytometry.com/how-to-compensate-a-4-color-flow-cytometry-experiment-correctly/

# Transformation




As you can see on the left, the data is compensated but the display is troublesome. The reason the data is displayed incoherently is because it has yet to be *transformed*.

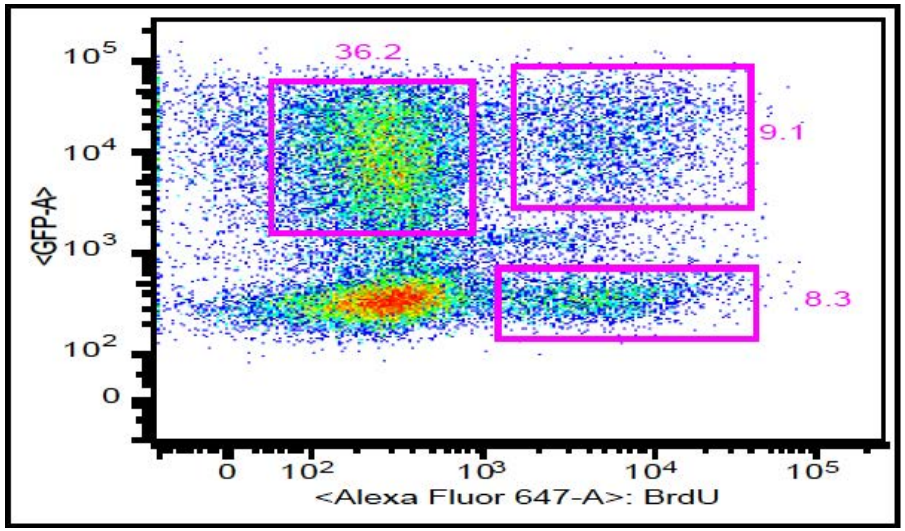
Transformation allows the full spread of the data to be visualized, while removing events off the axis. As shown on the right, when the correct transformation is applied, the data around 'zero' on both the Y-axis and X-axis is re-plotted. **Now the data is shown WITHOUT being compressed against these axes.**

https://expertcytometry.com/how-to-compensate-a-4-color-flow-cytometry-experiment-correctly/

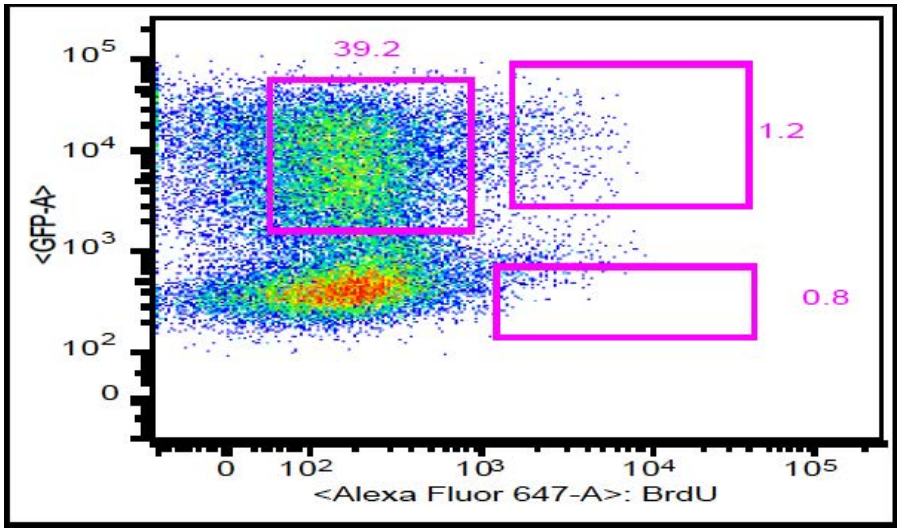
# Tricky stains
# BrdU

Fc-Block + EMA
Surface staining
EtOH → destroys APC, APC-Cy7!
1% PFA
100U DNaseI
Intracellular staining + anti-BrdU ab over night

60min PFA




10min PFA



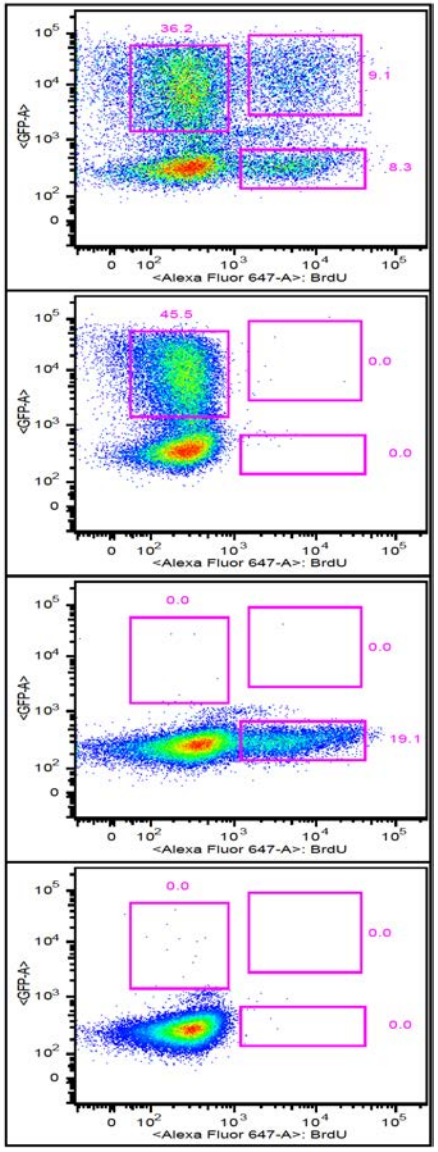
# Tricky stains
# BrdU + GFP

GFP+
BrdU injected

GFP+
PBS injected

GFP -
BrdU injected

GFP -
PBS injected




Fc-Block + EMA

Surface staining

---IF staining GFP+ cells: 5min 1% PFA at RT

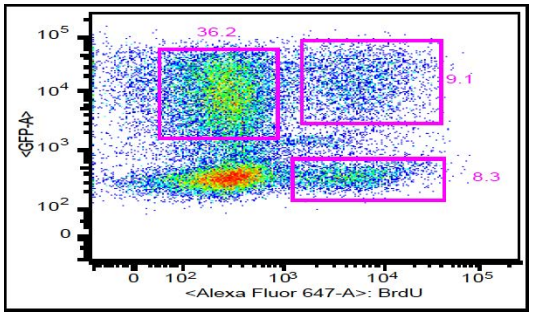
EtOH

PFA

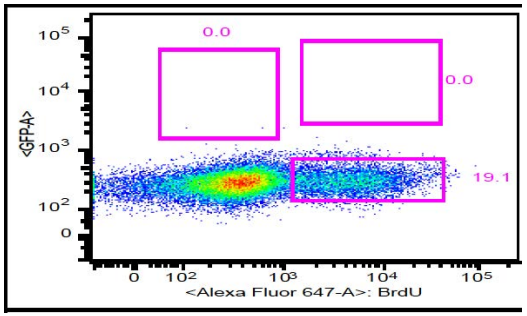
DNaseI

Intracellular staining + anti-BrdU ab

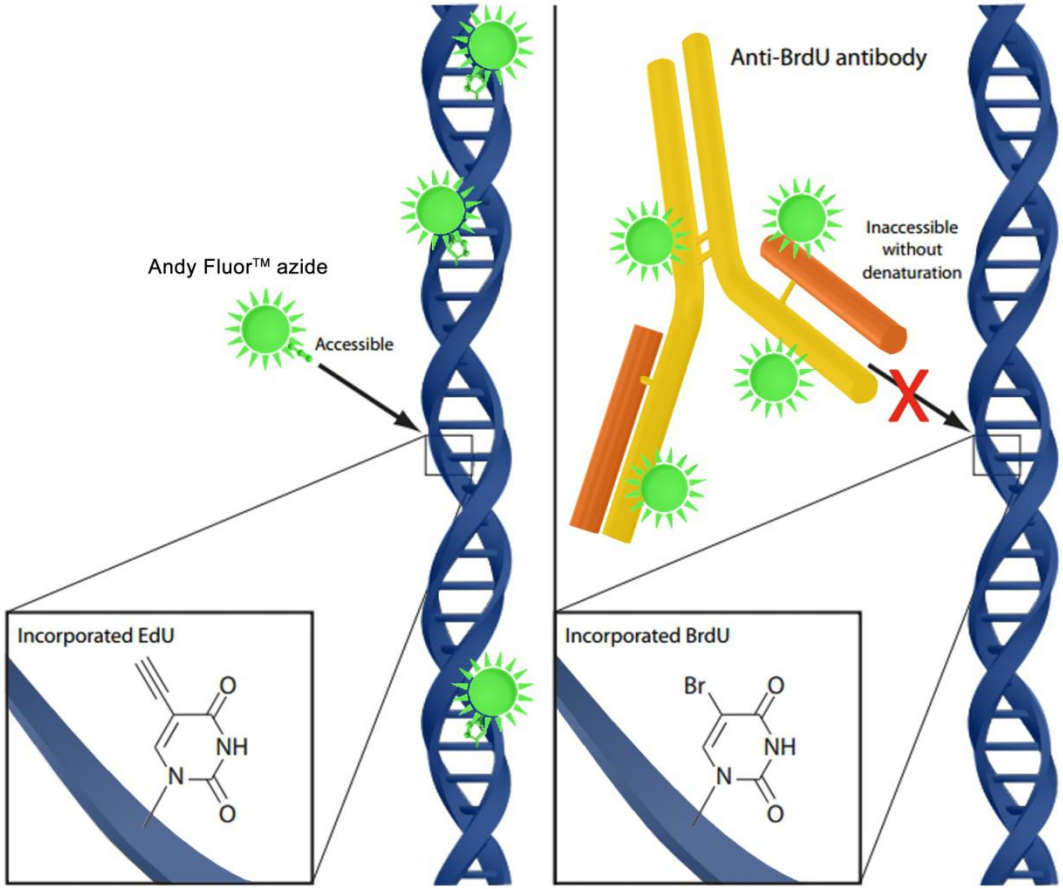
+ prefix




- prefix

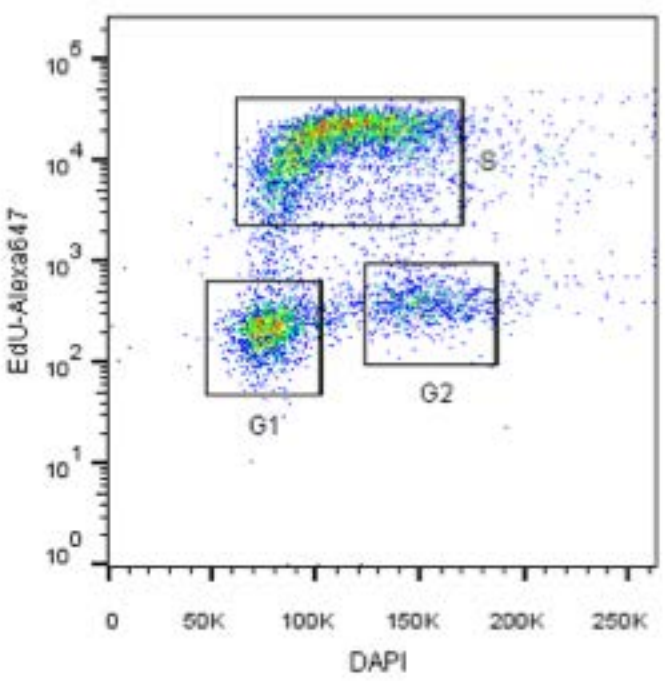


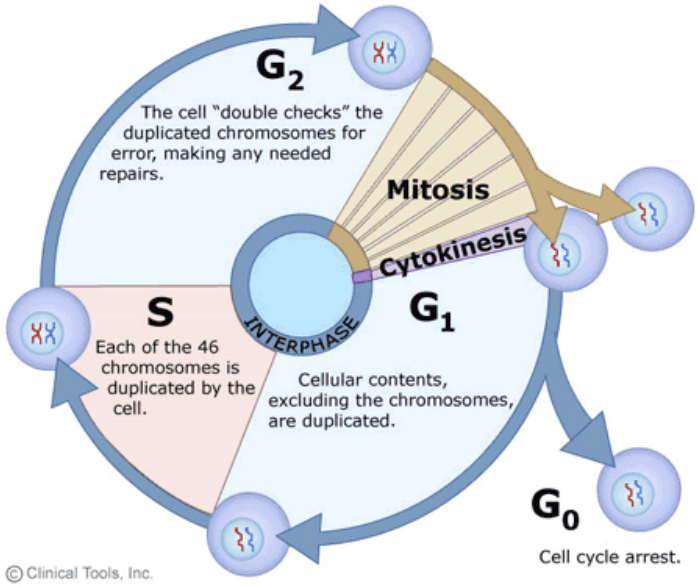
# BrdU vs EdU


Andy Fluor™ azide
Accessible
Anti-BrdU antibody
Inaccessible
without
denaturation
X
Incorporated EdU
O
NH
N
O
Incorporated BrdU
Br
O
NH
N
O

# BrdU vs EdU
# Cell cycle analysis

EdU is very expensive … but you can buy it from Carbonsynth LLC (1g for $240)
ThermoFischer charges $459 for 50mg (you can also buy 5g for $4.830 there ☺)
So EdU and BrdU are around same prize given that you need the expensive aBrdU antibody or the EdU kit



# Declogging
# FIRST RUN BLEACH+WATER & “PRIME

# Declogging

# Declogging

# Declogging

REQUIRES
TRAINING by FlowCore!!!!!

# Cleaning

Run 5 min bleach after your experiment and record it

Run 5 min water and record it

# Cell Sorting

Prepare samples at around **$30x10^6$ lymphocytes per ml**
You can sort into plates (up to 384) and various tubes

| | |
|---|---|
| 1.5ml | 4 streams |
| 5m | 4 streams |
| 15ml | 2 steams |

(50ml no holder but can just put in rack)

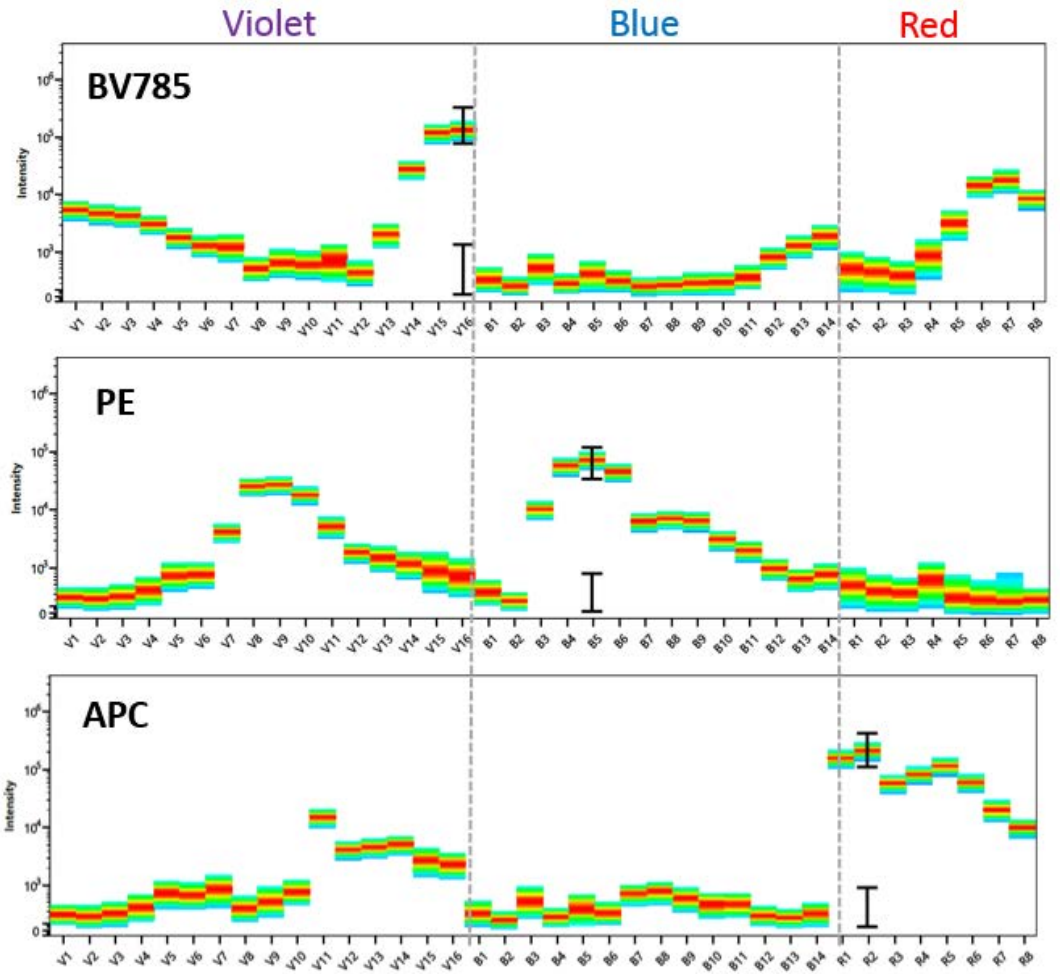
Collection tubes can be cooled – let operator know in advance

Block collection tubes with protein to prevent sorted cells sticking to plastic

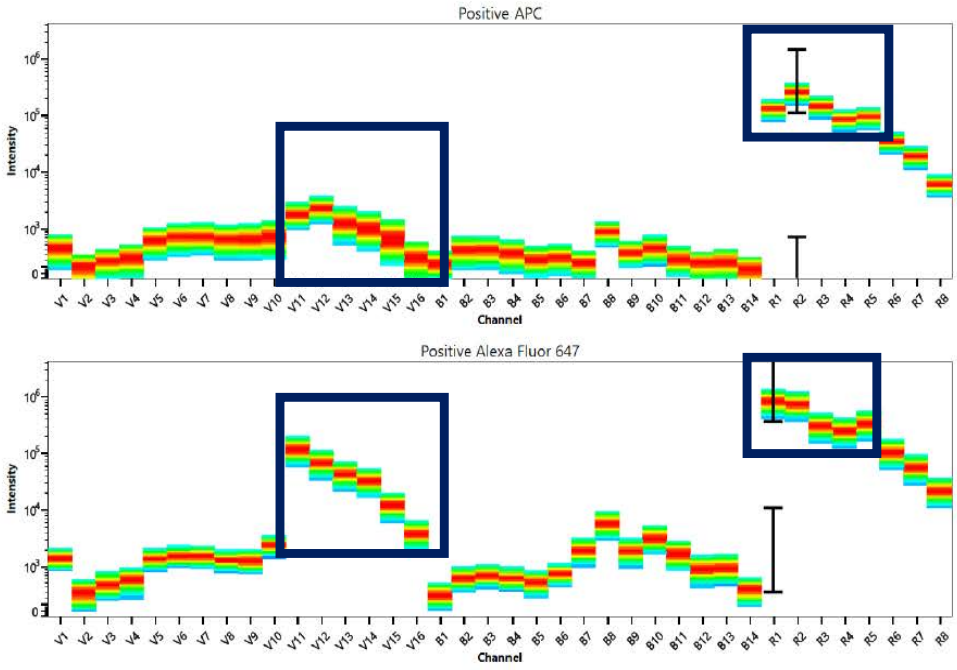
# Aurora

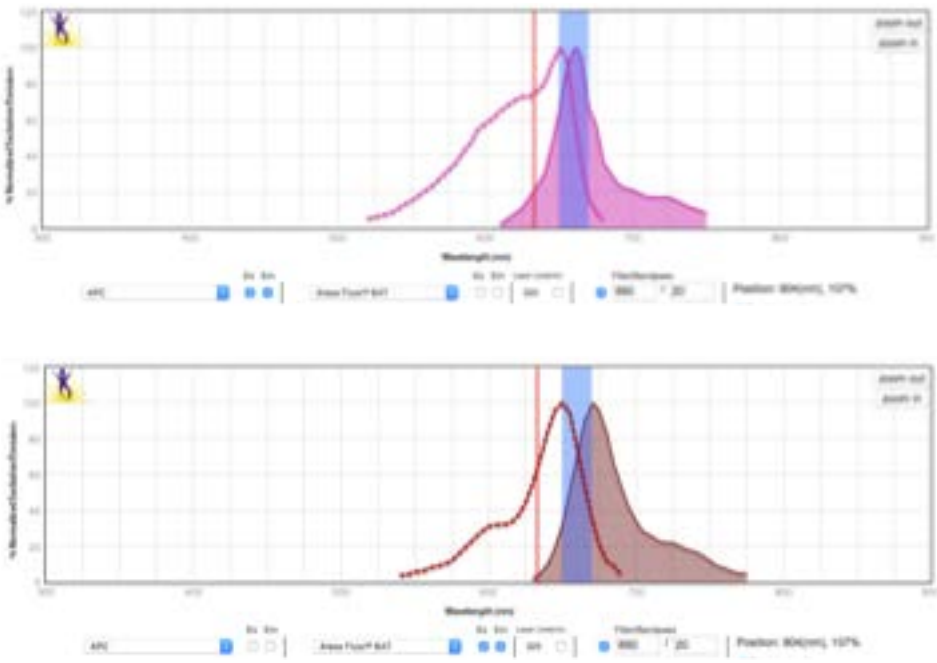
## What Is a Full Spectrum Signature?

- The Aurora is capable of measuring the entire emission spectra of fluorescent dyes excited by the installed lasers.
- Emission spectra excited by the Violet, Blue, and Red lasers are measured from the laser line through the infrared region.
- Full spectrum capture enables the use of novel unmixing algorithm for data analysis.



# Aurora vs regular cytometer



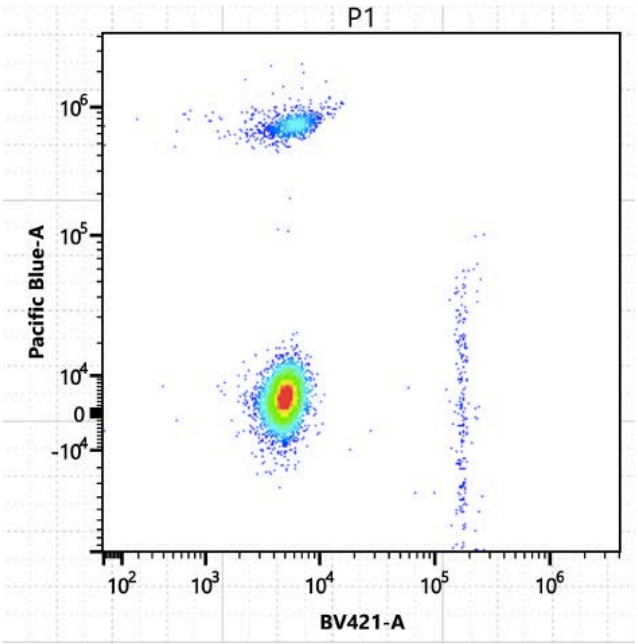



APC and Alexa 647 have different full spectrum

Can be separated on Aurora

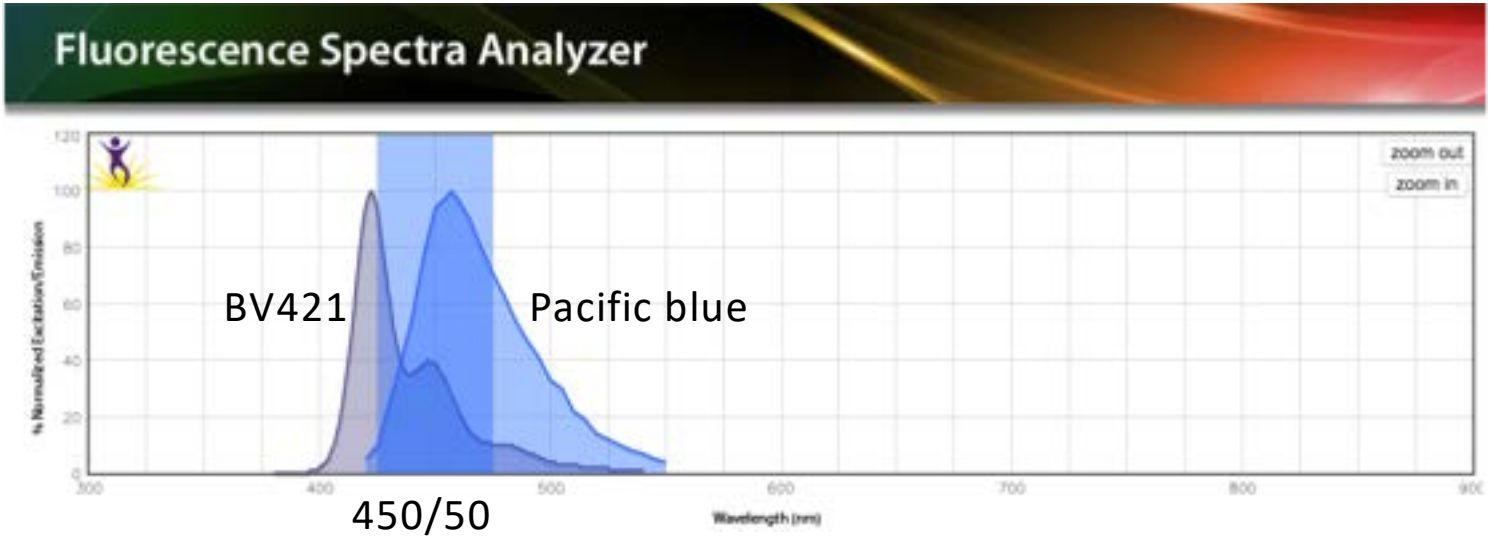
Cannot be separated on regular cytometer

# Aurora




pacific blue and BV421 works together on Aurora

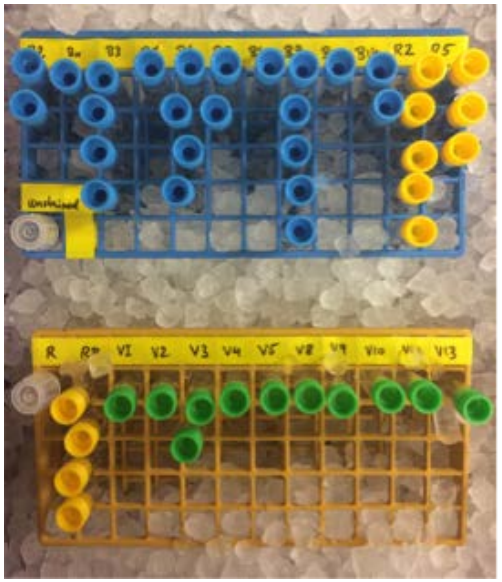
Same channel on regular cytometer



# Aurora
# Multi multi color flow

| | |
|---|---|
| B2/ Bx | BD Horizon BB515<br>**AF 488**<br>FITC |
| B3 | **AF 532** |
| B5 | **PE** |
| B6 | PE-CF594<br>**PE/Dazzle 594**<br>PE-efluor 610<br>Pe-Texas Red<br>PE-AF610 |
| B8 | **PE-Cy5** |
| B9 | PE-Cy5.5<br>PerCP<br>**PerCP-Cy5.5**<br>BB700 |
| B10 | PE-AF700<br>**PerCP-eFLuor 710** |
| B14 | **PE-Cy7** |
| R2 | **APC**<br>eFluor 660<br>AF 647 |
| R4 | APC-Cy5.5 |
| R5 | APC-R700<br>**AF700** |
| R | **Zombie NIR** |
| R8 | **APC/Fire 750**<br>APC-Cy7<br>APC-eFluor 780 |

| | |
|---|---|
| V1 | **BV421** |
| V2 | Super Bright 436 |
| V3 | eFluor 450<br>BD V450<br>**pac blue** |
| V4 | BV480 |
| V5 | **BV510**<br>eFluor 506<br>BD V500 |
| V8 | **pacific orange** |
| V9 | **BV570** |
| V10 | Super Bright 600<br>**BV605**<br>eVolve 605<br>Qdot 605 |
| V11 | Super Bright 645<br>**BV650**<br>eVolve 655<br>Qdot 655 |
| V13 | Super Bright 702<br>Qdot 705<br>**BV711** |
| V14 | **BV750** |
| V16 | **BV786**<br>Qdot 800 |

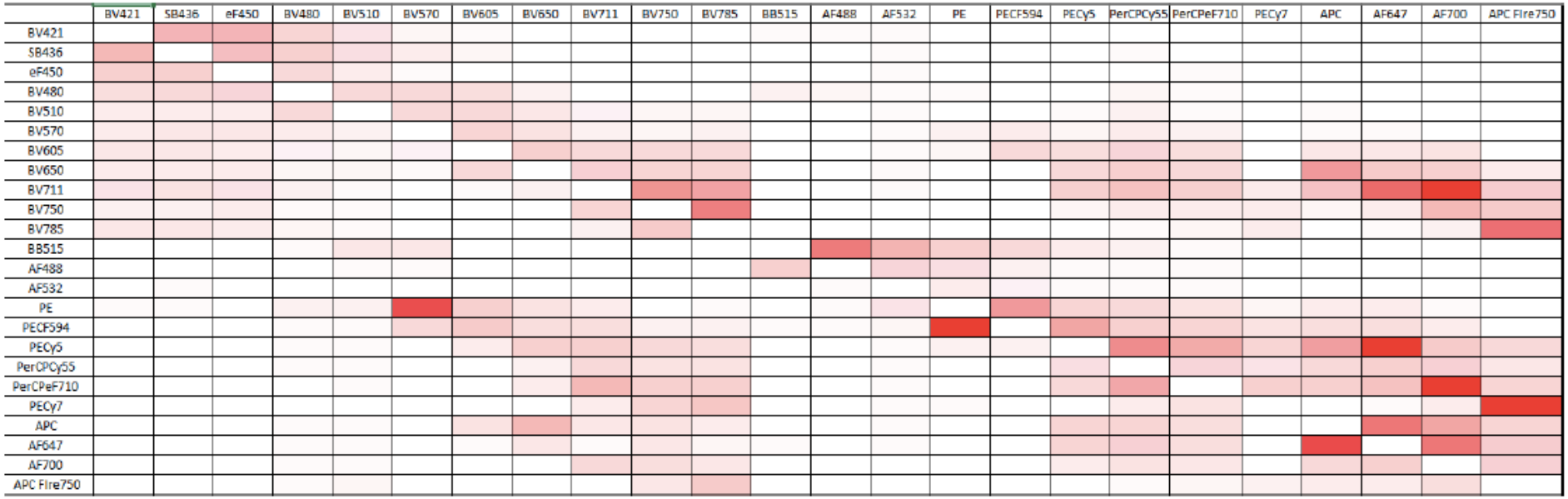


**Validated by Cytek**

**My favorite panel**

# Aurora
# Multi multi color flow

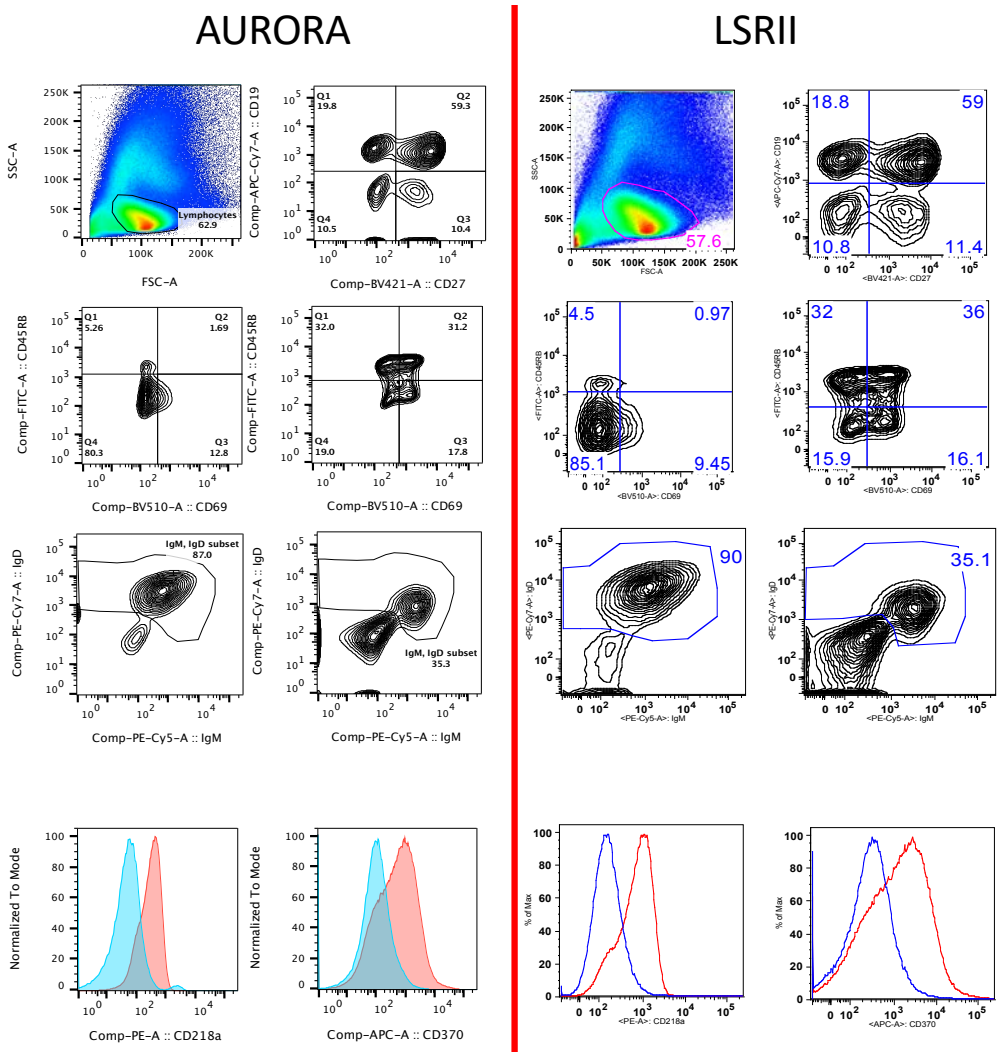
## Spread matrix for 24 Fluors that can be use in combination



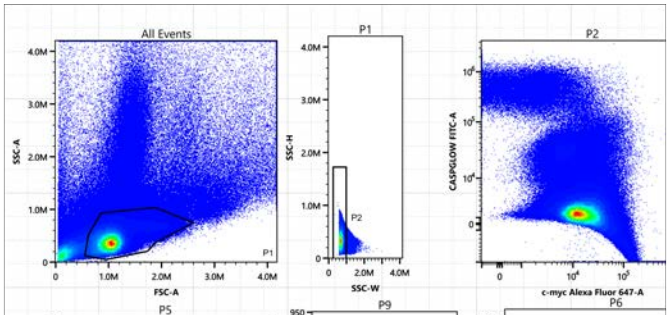
| | BV421 | SB436 | eF450 | BV480 | BV510 | BV570 | BV605 | BV650 | BV711 | BV750 | BV785 | BB515 | AF488 | AF532 | PE | PECF594 | PECy5 | PerCPCy55 | PerCPeF710 | PECy7 | APC | AF647 | AF700 | APC Fire750 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BV421 | | | | | | | | | | | | | | | | | | | | | | | | |
| SB436 | | | | | | | | | | | | | | | | | | | | | | | | |
| eF450 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV480 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV510 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV570 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV605 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV650 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV711 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV750 | | | | | | | | | | | | | | | | | | | | | | | | |
| BV785 | | | | | | | | | | | | | | | | | | | | | | | | |
| BB515 | | | | | | | | | | | | | | | | | | | | | | | | |
| AF488 | | | | | | | | | | | | | | | | | | | | | | | | |
| AF532 | | | | | | | | | | | | | | | | | | | | | | | | |
| PE | | | | | | | | | | | | | | | | | | | | | | | | |
| PECF594 | | | | | | | | | | | | | | | | | | | | | | | | |
| PECy5 | | | | | | | | | | | | | | | | | | | | | | | | |
| PerCPCy55 | | | | | | | | | | | | | | | | | | | | | | | | |
| PerCPeF710 | | | | | | | | | | | | | | | | | | | | | | | | |
| PECy7 | | | | | | | | | | | | | | | | | | | | | | | | |
| APC | | | | | | | | | | | | | | | | | | | | | | | | |
| AF647 | | | | | | | | | | | | | | | | | | | | | | | | |
| AF700 | | | | | | | | | | | | | | | | | | | | | | | | |
| APC Fire750 | | | | | | | | | | | | | | | | | | | | | | | | |

*To read this table: fluor in the row impacts the one in the column. Red means the fluor in that row has significant spread into the dye in the column (for example PE into BV570). Areas in bright pink and red is where more attention to panel design is needed.*

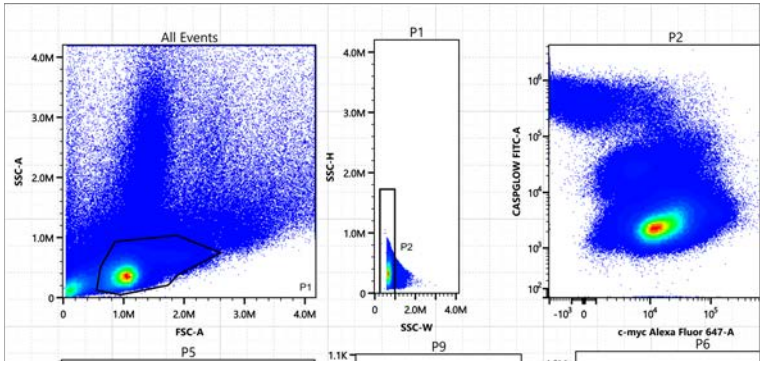
# Aurora vs LSRII



# Aurora – unmixing
# You can change your raw data afterwards




Had bad (=way too bright single color controls). Recorded better single color controls next day and unmixed again



# Thanks for your Attention

## Questions?