Rules in Rare Event Acquisition
An Overview

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DEFINITION

'**Cytometric Rare Events**' can be defined as cell populations represented at ≤ 10^{-3} over the total events (i.e. ≤ **0,1%** or ≤ **1 / 1,000** total events)

In this presentation we will see how Multicolor Flow Cytometry can effectively and accurately manage extremely rare event analyses down to 10^{-5} or ≤ **0,001%** or ≤ **1/ 100,000**.

FLOW CYTOMETRIC ANALYSIS OF RARE CELL EVENTS IS AN ESSENTIAL TOOL IN A NUMBER OF CRUCIAL CLINICAL APPLICATIONS

- Minimal Residual Disease (MRD) Analysis in Leukemias and Myeloma.
- High-Resolution Detection of PNH Clones.
- Analysis of Feto-Maternal Hemorrhage (FMH).
- Enumeration of functional B-Cell Subsets in Anti-CD20 Therapies.
- Enumeration of Antigen-Specific T-Cells.
- Enumeration of Residual White Cells in Leukoreduced Blood Products.
- Detection and Enumeration of Circulating Endothelial Cells (CEC).
- Detection and Characterization of Infrequent Cell Populations (i.e. *Dendritic Cells, Stem Cells and Precursors, Circulating Neoplastic Cells...*)

RARE EVENT ANALYSIS BY FLOW CYTOMETRY - TECHNICAL PREREQUISITES

- Ensure a careful cleaning of the fluidic system and particle-free reagents.
- Ensure the maximal specificity of the staining protocol (*multicolor analysis*).
- Prepare a cell-rich sample to collect a high number of cell events.
- Use the 'Fluorescence Minus One' – FMO approach to set analysis windows.
- Set a well designed gating syntax aimed at eliminating non-specific events.
- Event numbers are in many cases more important than the number of colors !
- Acquire the highest possible amount of total cell events (*denominator*).
- Acquire the highest possible amount of relevant cell events (*numerator*).
- Master the Lower Limits of Detection (LOD) and Quantification (LOQ).

RARE EVENT ANALYSIS - Possible Pitfalls and Assay Limitations

- Dirty fluidics or sample carryover → Non-specific events are acquired.
- Whole blood Stain-and-Lyse → More non-specific events than with Bulk Lysis.
- Cell-poor samples require concentration before and/or during analysis .
- Excess fluorescence spillover generates a lot of false signals.
- Gating syntax has a great impact → Elimination of doublets, Gating out fluidic perturbations, Dump channels for undesired events, FSC/SSC Backgating, aiming at the 'virtual zero events' in the acquisition window with neg control.
- Cell ***Denominator*** → MILLIONS of clean cell events are required.
- *Relevant Cell Population* (***Numerator***) → Best >100; LOD>20/30; LOQ>50 events.
- Experimental conditions are VERY DIFFERENT from real life.

RARE EVENT ANALYSIS BY FLOW CYTOMETRY

A Very Basic Problem: The Interference by Non-Specific Events

- In every flow cytometric analysis a number of **Non-Specific Events** are normally collected (i.e. parasite cell- and non-cell events falling into the relevant event window), which pose **a problem for the Signal-to-Noise Ratio**.

- Such non-specific cell events are normally of little practical importance when they are limited to <1% and large cell populations are analyzed (i.e. CD4+ T cells).

- However, in case of Rare Event Analysis the non-specific cell events can often outnumber the relevant cell frequency, making the count totally unreliable.

- **Typical non-specific events are caused by**: A dirty fluidic system, Platelets and especially giant platelets, Cell debris, Complex indirect and intra-cytoplasmic staining procedures, Badly conjugated antibodies, Fluorescence spillover, Weak gating syntax and many more...

- Methods to be used to minimize the generation and the acquisition of non-specific events are brilliantly described in this paper: → **see next slide**

Clinical Cytometry 2009; 76B: 355 - 364

Review Article

Considerations for the Control of Background Fluorescence in Clinical Flow Cytometry

Ruud Hulspas,[1*] Maurice R.G. O'Gorman,[2] Brent L. Wood,[3] Jan W. Gratama,[4] and D. Robert Sutherland[5]

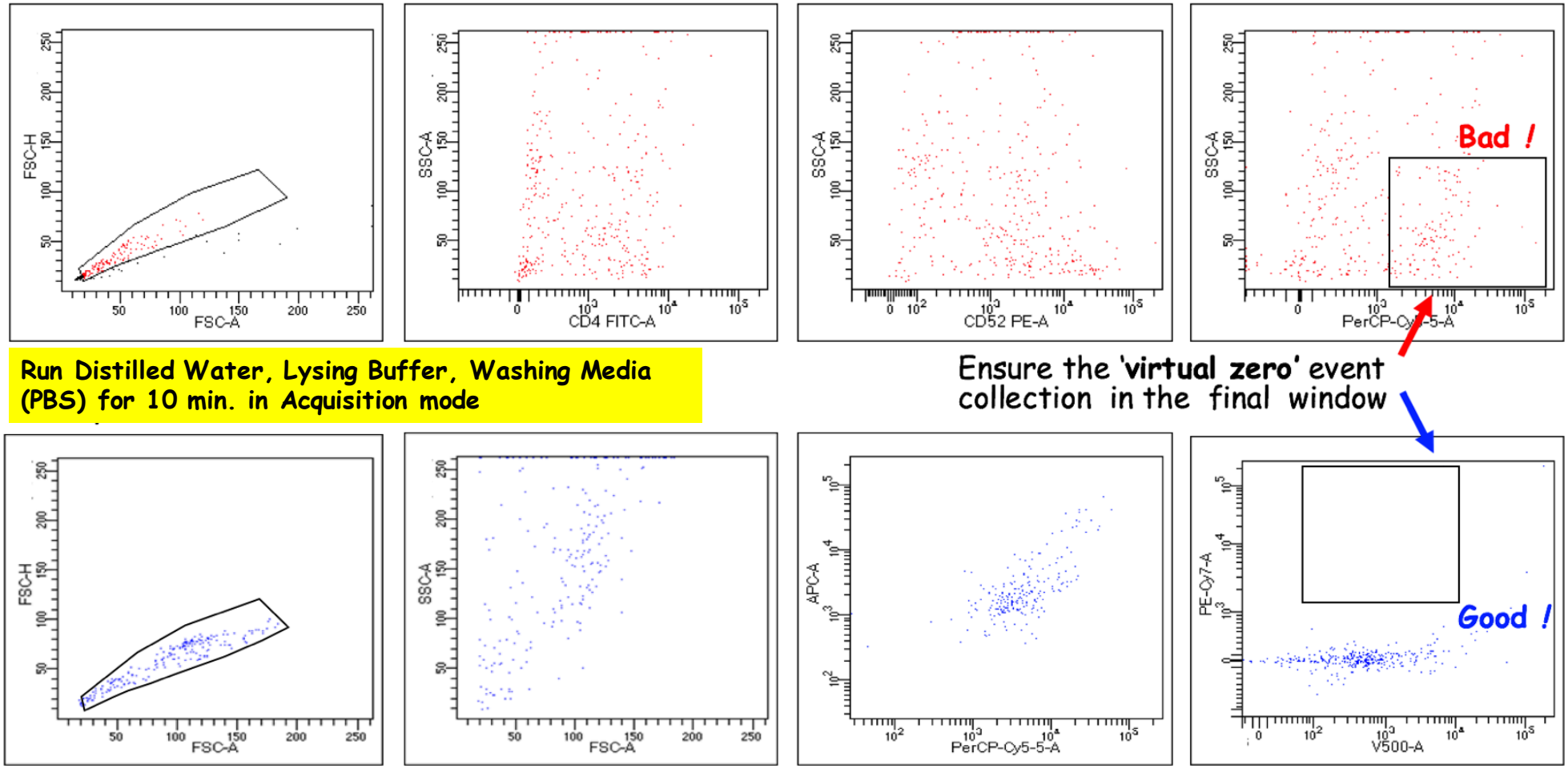
This useful paper reviews extensively all the factors that generate autofluorescent and background non-specific events, and illustrates the ways to avoid or minimize them.

The following topics, which deeply influence Rare Event Analyses are covered:

- Autofluorescence: definitions and examples
- Background caused by spectral overlap and fluorescence spillover
- The many undesirable ways a conjugated monoclonal can bind to cells
- The never ending story of isotype controls
- The isoclonic controls
- The proper setting of internal negative controls
- The mandatory use of Fluorescence-Minus-One (FMO) controls
- **A series of practical recommendations is given**

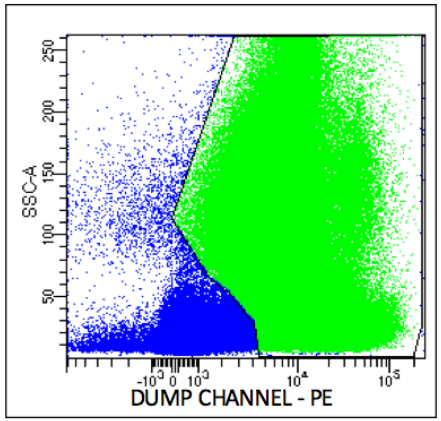
Please Take a Look Before Tackling Rare Event Analysis

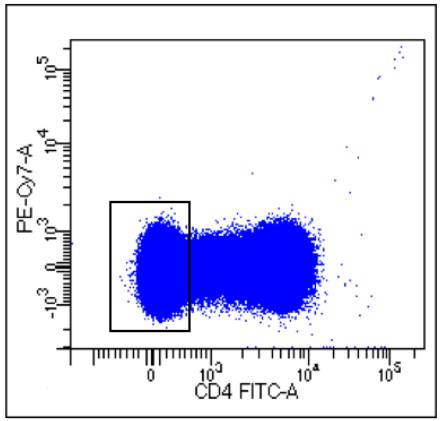
Checking the Cleanliness of the Fluidic System and of the Media and Buffers – Carryover and Stray Events

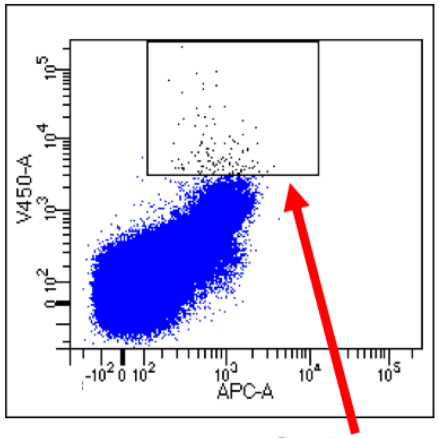


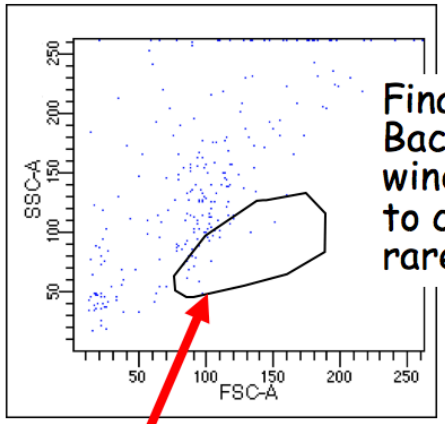
If stray events are collected in the final window, perform a long instrument cleaning and/or prepare new fresh media

Checking the Cleanliness of the Overall sample Preparation Procedure



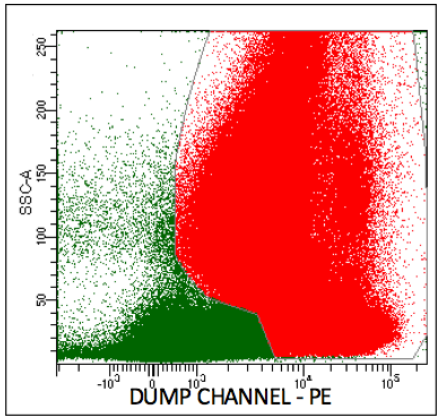


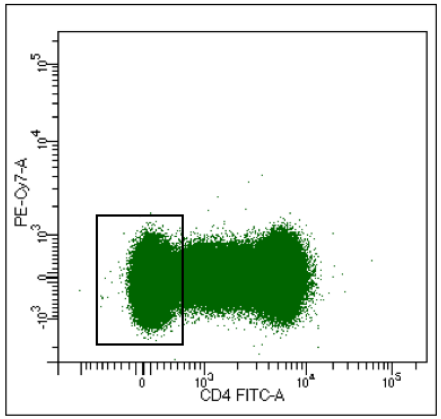


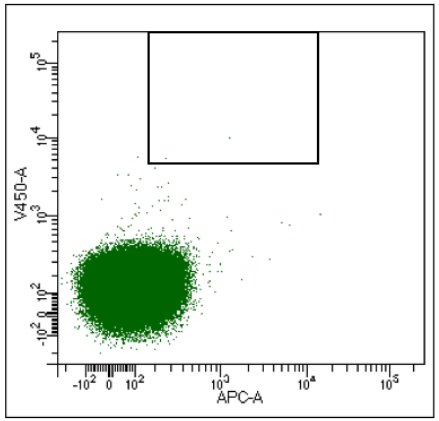


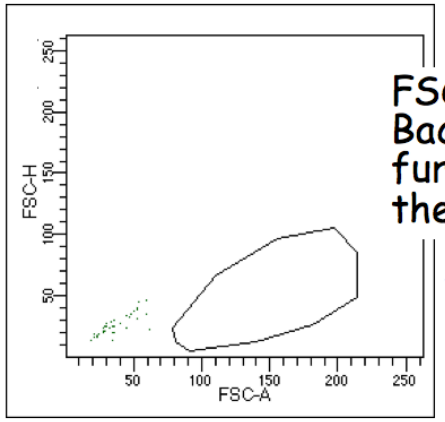
Final FSC/SSC Backgating window to collect rare events

A **BAD** example is shown here:





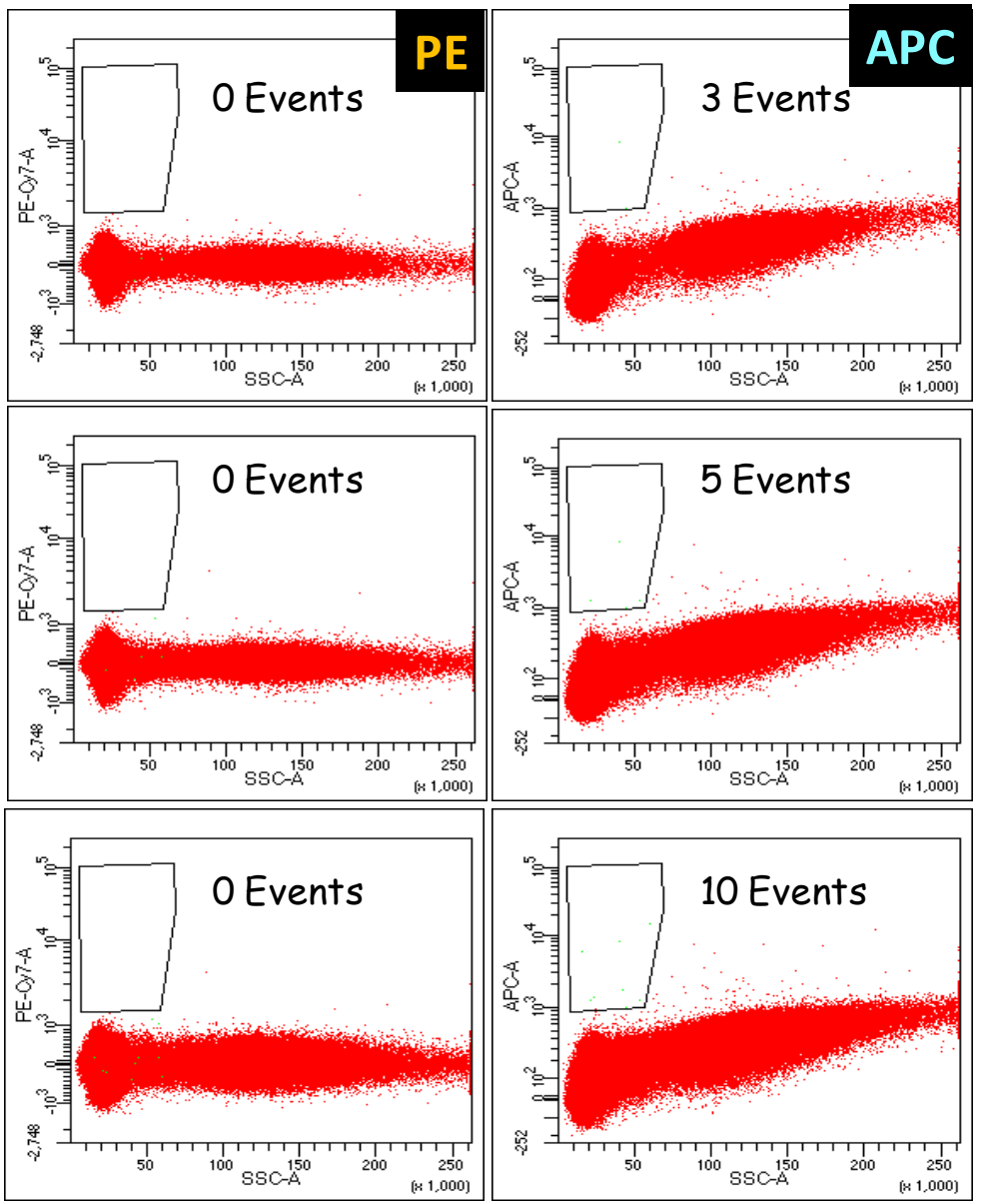




FSC / SSC Backgating further helps the cleaning

If stray events persist in the backgating window, check also lysing/washing media and centrifugation speed/time.

POSITIVE
Background Events depend from the total number of acquired cells and vary according to fluorochrome channel



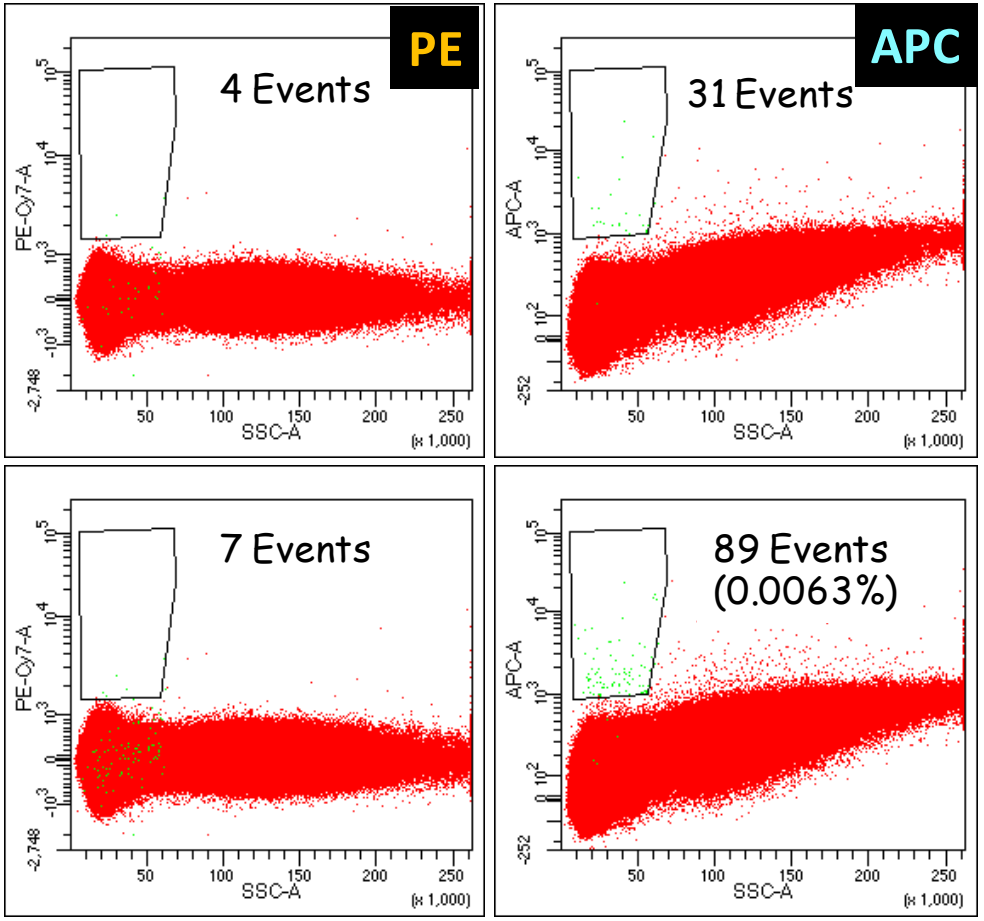
Acquisition:
150,000 Events

Acquisition:
250,000 Events

Acquisition:
350,000 Events

POSITIVE
Background Events:

Some color channels seem 'cleaner' than other ones (due to photon energy statistics).

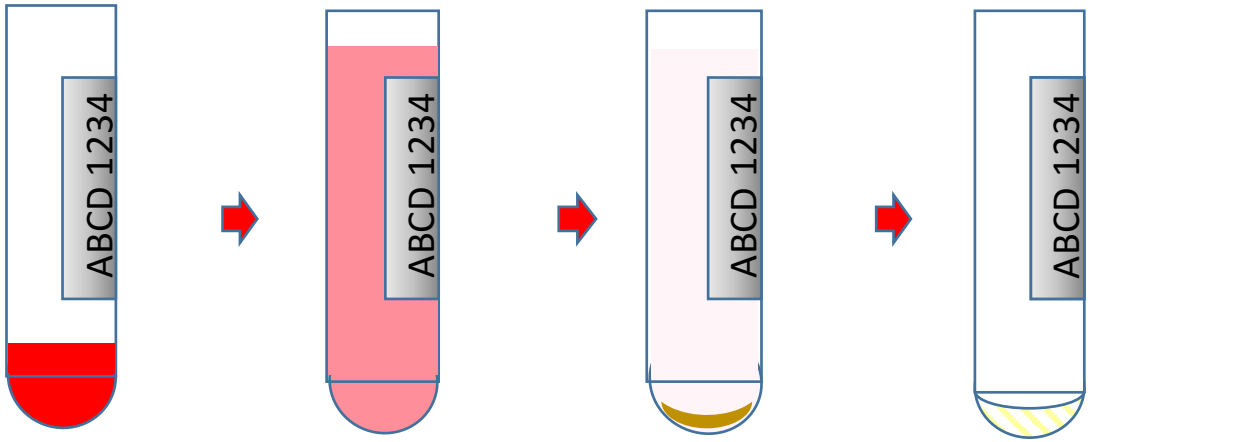


Acquisition:
600,000 Events

Acquisition:
1,400,000 Events

(LLOQ= 0.0035%)

Bulk Lysis: An Effective Way to Reduce Plasma and Platelet Interferences and to Concentrate Cells in Paucicellular Samples – **Ideal for Rare Event Analyses**



1-2 mL Blood or Dissociated Bone Marrow Suspension in a 10-12 mL tube.

Add 8-10 mL of Ammonium Chloride Lysing. Gently mix and incubate at RT° for 10-15 min.

Spin and wash twice with 10 mL PBS + 0.5% BSA.

Resuspend the final cell pellet in 200-300 µL PBS (Best if supplemented with 0.5% BSA)

As compared to the conventional single-tube Stain-and-Lyse technique **the Bulk Lysis procedure** removes plasma and the majority of platelets (greatly reducing interferences)

AND

Allows the **concentration** of the initial sample by a factor of **3-4 to 10**, depending on the sample and diluent volumes used.

Bulk Lysis is recommended in Leukemia/Myeloma MRD studies and in high-resolution PNH analysis

- Flores-Montero J. Leukemia 2017; 31: 2094-2103.
- Dahmani A. Am J Clin Pathol 2016; 145: 407-417.
- Kumar S. Lancet Oncol 2016; 17: e328-346.

Ordinary Whole Blood Staining Increases Background 'PNH' Monocyte Events
Bulk Lysis and Wash Before Staining Reduces Background Events by > 1Log

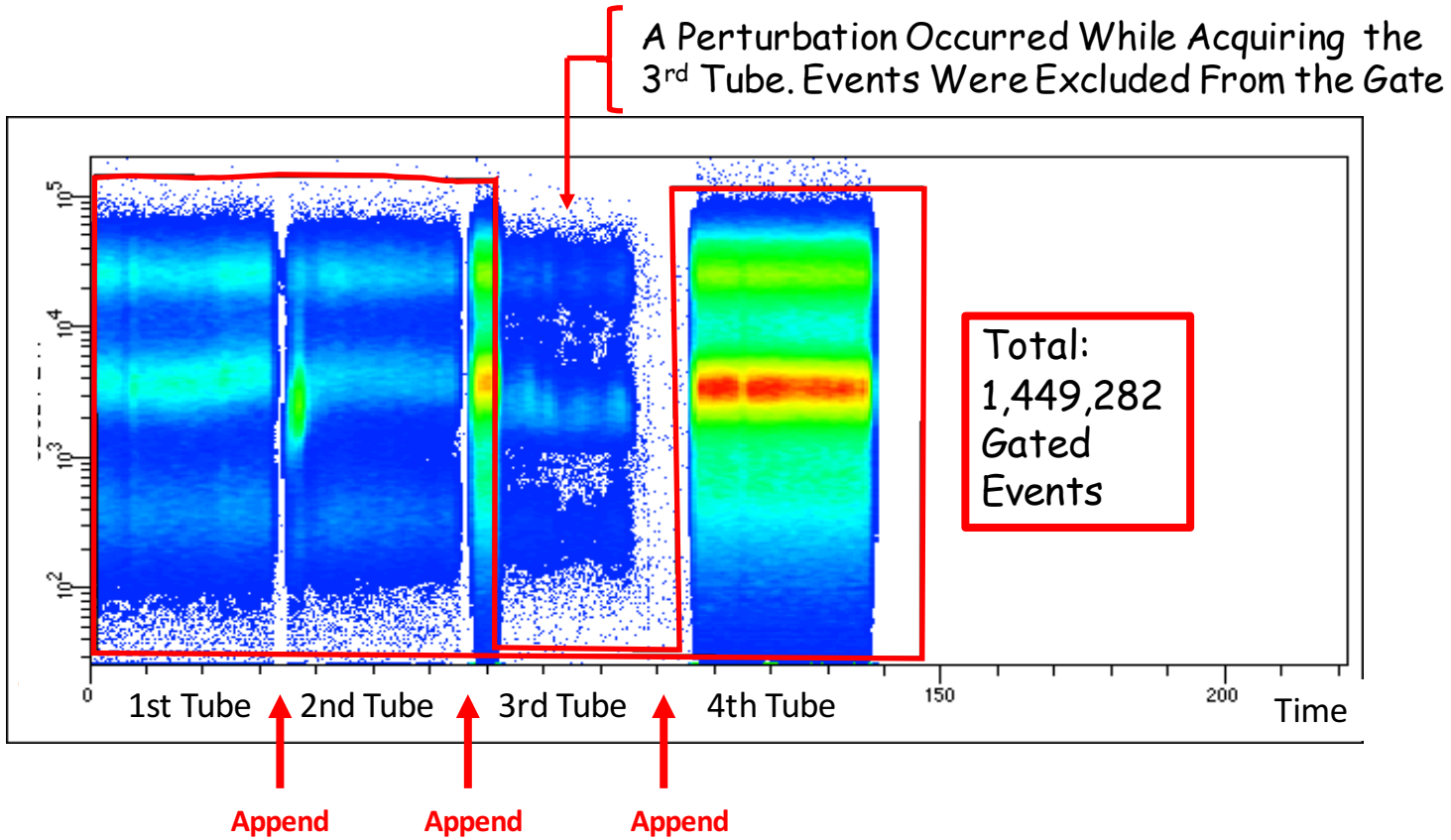
Cellular Background and Limits of Detection Determined on 20 Healthy Subjects

Cell Type/Parameter	Whole-Blood Staining	Washed Whole Blood	Lyse Before Stain
Granulocytes			
Mean No. of FLAER-negative cells among 250,000 granulocytes	2.16	1.7	1.66
No. + 5 SD	12.4	10.7	8
Limit of detection, %	0.0049	0.0043	0.0032
Monocytes			
Mean No. of FLAER-negative cells among 10,000 monocytes	3	0.62	0.46
No. + 5 SD	40.4	3.4	1.9
Limit of detection, %	0.41	0.034	0.019

- Bulk lysis, wash and stain procedure removes the interfering effects of plasma and a lot of disturbing platelets.
- FLAER fluorescence intensity **increases remarkably with bulk lysis.**

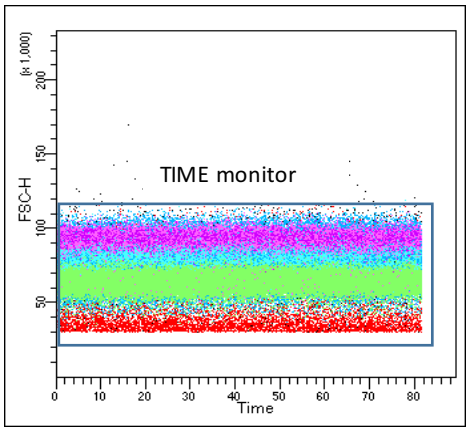
Dahmani A. Am J Clin Pathol 2016; 145: 407 - 417

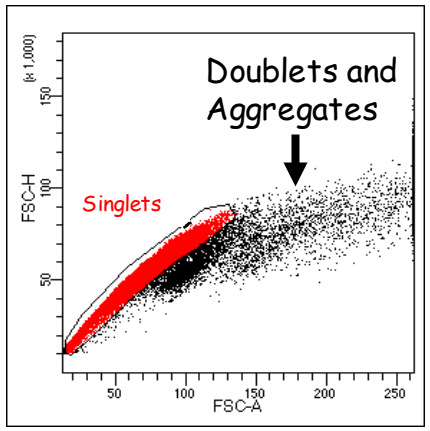
Tricks to Further Increase Cell Collection: Sample Replicates and Instrument APPEND Function



Using identical sample replicates in multiple tubes may greatly increase the collection of cells. Taking advantage from the APPEND instrumental function, the temporary stop of the acquisition flow is possible, so that new tubes can be inserted and events merged in a single datafile.

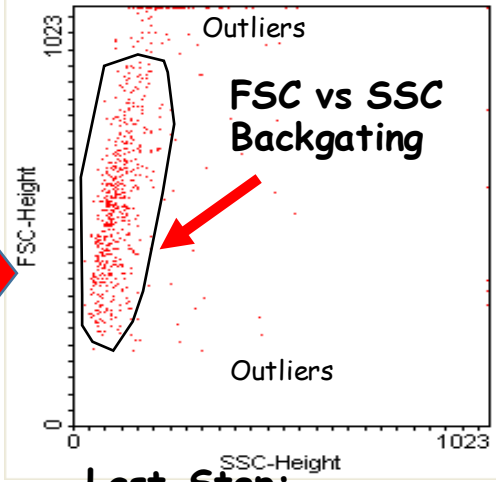
Setting the Right Gating Syntax for Rare Event Analysis: General Rules





Your Own Experiment-Specific Gating Protocol

(Dump channel optional)



First Step: Include the TIME parameter

- To monitor the regularity of the long acquisitions.
- To gate out perturbations.

Second Step: FSC-A vs FSC-H Doublet Discriminator

- To include only cell singlets into analysis.
- Greatly reduces artifacts caused by aggregates.

Third Step: Your Own Logical Gating Protocol

- Any sequential procedure to include relevant cells and to exclude undesired cell populations.

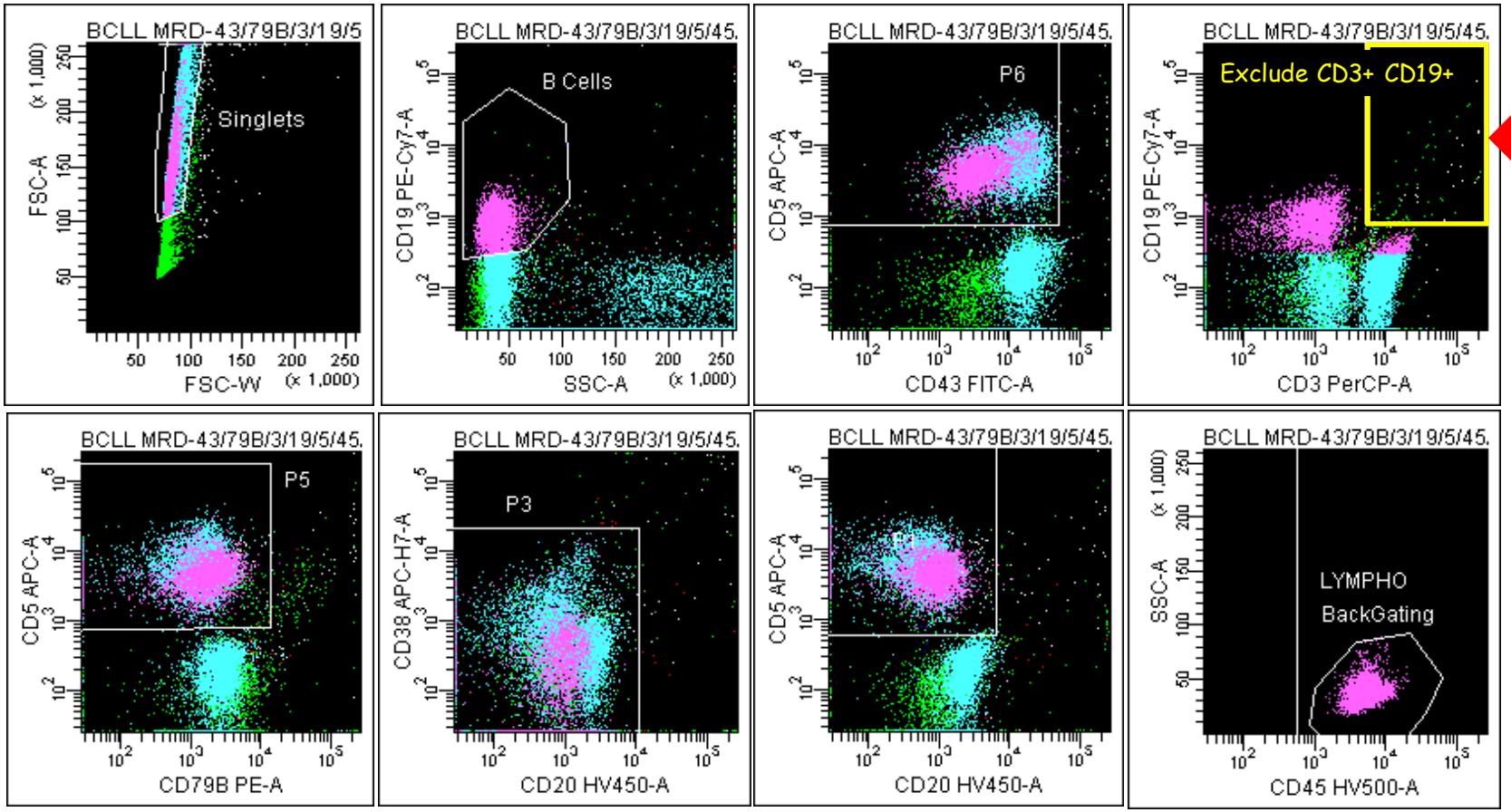
Last Step: FSC vs SSC Backgating

- Fluorescence does not tell you all, and can cheat you.
- Check the rare cell physical parameters consistency.
- Efficiently eliminates parasite events and outliers.

These conservative gating rules can be widely applied to **any** Rare Event Analysis protocol using multicolor fluorescence, ensuring a very efficient elimination of artifacts and parasite events.

Setting the Right Gating Syntax for Rare Event Analysis: A Special Case

The **ERIC** Standardized Approach to B-CLL Minimal Residual Disease, **1-tube 8-Colors**:
CD43 FITC/ CD79b PE/ CD3 PerCP/ CD5 APC/ CD19 PE-Cy7/ CD38 APC-H7/ CD20 V450/ CD45 V500.



As anyone may know, **CD3+ CD19+** Cells do not exist.

However, in complex multicolor procedures a few artefactual CD3+ CD19+ events can be generated by non-specific antibody binding.

Despite such false events are detectable in low numbers, in B-CLL MRD studies they may influence the analysis results **if not properly gated out**.

ERIC Specification for the Detection of B-CLL Cells in a Normal Background

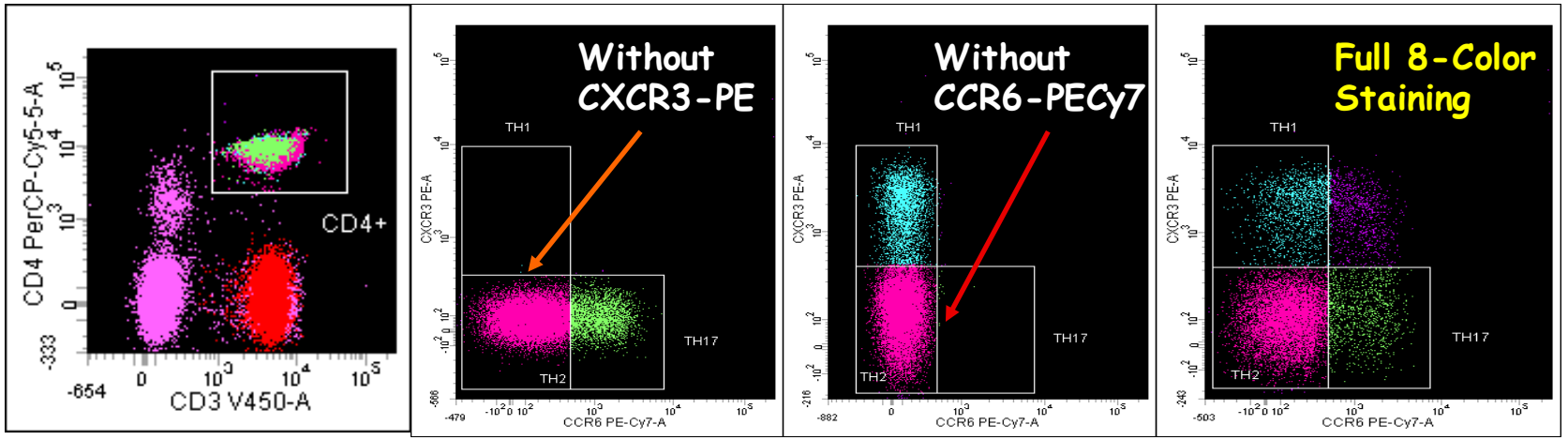
- CD3 may be informative if only 500,000 events or fewer have been acquired and the result is within 0.5log of the 0.010% threshold *(→ MRD threshold)*
- CD3 is uninformative for results that are above 2x the limit of quantitation (50 events) or below the limit of detection (20 events).
- For example if 500,000 events are acquired and there are fewer than 20 events (<0.0040% CLL) or more than 100 CLL-phenotype events (>0.020% CLL), the inclusion of CD3 would have no significant impact on the results but if there are 20-100 CLL-phenotype events then the inclusion of CD3 may adjust the result below the 0.010% threshold (e.g. a result of 0.012% without analysis of CD3 may equate to a result of 0.0080% when CD3 is included in the analysis).

CD3 is MANDATORY when > $2x10^6$ events are acquired, to ensure LOQ <0.005%

Courtesy of Andy Rawstron, ERIC Workshop, Stresa 2019

Setting the Windows to Collect Relevant (More or Less Rare) Events
Using The Fluorescence-Minus-One Approach (FMO)

CD45-FITC/ **CXCR3**-PE/ **CD4**-PerCP-Cy5.5/ **CCR6**-PE-Cy7/ **CD38**-APC/ **CD8**-APC-H7/ **CD3**-V450/ **HLA-DR**-V500

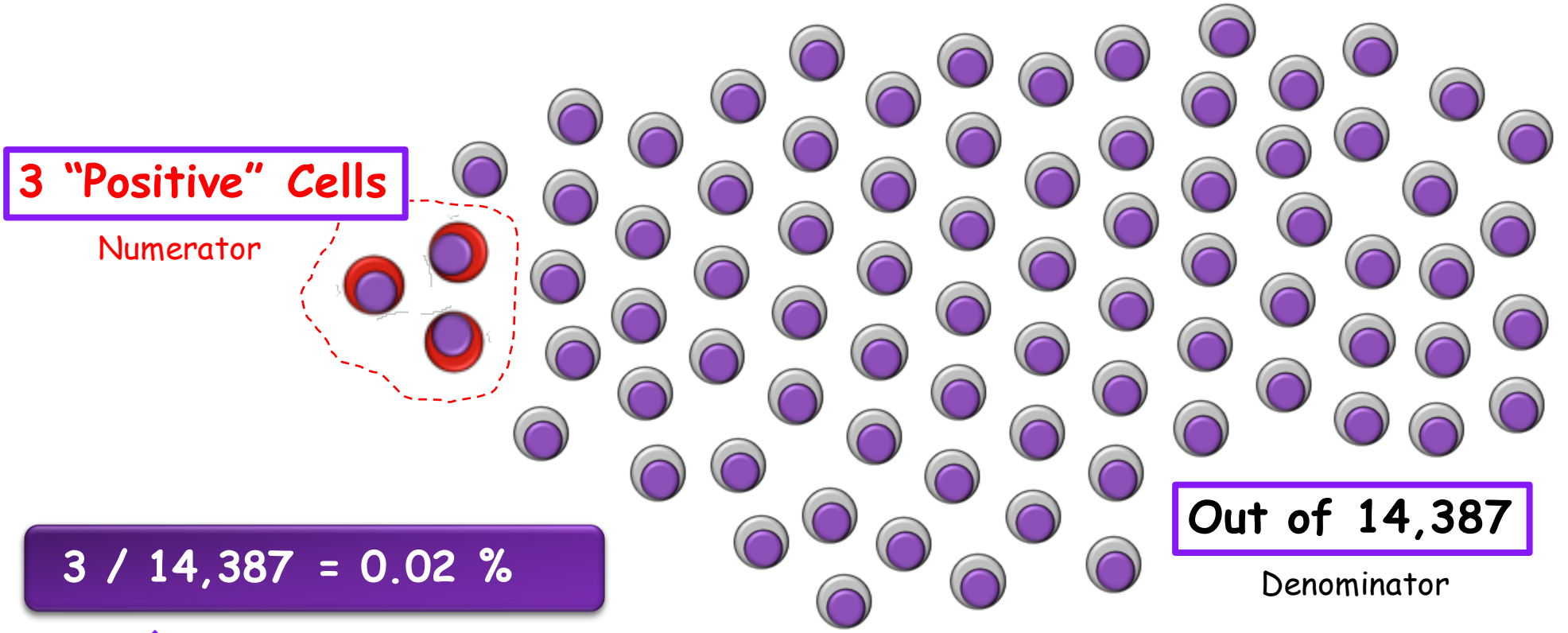


- In complex multicolor analyses the windows to collect the critical events should be always set on the basis of the FMO concept (*see: Roederer M. Cytometry 2001; 45: 194-205*).

- The FMO procedure takes into account all the emission spillovers affecting a given fluorescence channel (in the example PE for CXCR3+ T cells or PECy7 for CCR6+ T cells).

- The relevant (positive) event collection windows established with FMO are set more accurately than using the simple unstained control (*But always check the consistency of the FSC/SSC backgating thereafter*).

Counting cells is not like counting eggs: **The Issue of Cell Numbers**



3 / 14,387 = 0.02 %

Is it a correct approach? → **NO!**

Enumeration of rare cell events (i.e. < 10^{-3}) **is NOT a mere arithmetical calculation.** Stringent technical and statistical criteria are required (both for 'positive' and 'denominator').

Rare Event Analysis: A Little Statistics (1) *(sorry, but it's necessary!)*

Let's enumerate a total of **N events (Lymphocytes)**, of which **R** meet a certain criterion **(i.e. B Cells = 10%)**.

The **Proportion** of positives, **P=R/N**, indicates the **Probability** of a particular positive event of being observed, in this case **P = 10%** or **P = 0.1**.

Cells are randomly selected during analysis, so positives are classified with an intrinsic **Variance**, that can be calculated as: **Variance = Number N x P(1-P). In this case: Variance =N x 0.1 x 0.9, say =N x 0.09.**

The **Standard Deviation (SD)** is the square root of the Variance, and the **Coefficient of Variation (CV)** is calculated as: **CV = (SD x 100)/Variance.**

In the table below three experiments are shown, in which 1000, 5000 and 10000 lymphocytes are counted, each one with B cells = 10% of lymphocytes. The respective SD and CV are calculated accordingly.

Total N. Collected Lymphocyte Events →	1,000	5,000	10,000
Observed B Cells (10%) →	100	500	1000
With a SD of	9.5	21.2	30
And a CV% of	10.55	4.71	3.3

A CV ≤ 10% is considered the maximum acceptable variability for biological measurements

Rare Event Analysis: A Little Statistics (2) Going Into the Deep

Just remember: **P=R/N ; Variance = N x P(1-P) ; CV = (SD x 100)/Variance**

Frequency →	1%	1%	1%	0.1%	0.1%	0.01%	0.01%	0.01%
Events N	1,000	10,000	100,000	100,000	1,000,000	100,000	1,000,000	10,000,000
Positive R	10	100	1,000	100	1,000	10	100	1,000
Proportion P	0.01	0.01	0.01	0.001	0.001	0.0001	0.0001	0.0001
Variance	9.9	99.0	990.0	99.9	999.0	10.0	100.0	999.9
SD	3.15	9.95	31.46	9.99	31.61	3.16	10.0	31.62
CV	**31.46**	**10.05**	**3.17**	**10.0**	**3.16**	**31.62**	**10.0**	**3.16**

So, if you want to detect a rare cell population **RELIABLY** (say at an acceptable ***CV* ≤10%**) you **MUST collect ≥ 100 POSITIVE EVENTS in any case**, which means to run:

- **≥ 10,000** total events if the relevant cell population is at **1%** frequency
- **≥ 100,000** total events if the relevant cell population is at **0.1%** frequency
- **≥ 1,000,000** total events if the relevant cell population is at **0.01%** frequency

- *Total Events means '**CLEAN' Cell events**, devoided of any contaminant (i.e. debris, platelets, red cells...)*
- *Increasing the **Positive event number** improves the CV and the overall reliability of the measure.*

Rare Event Analysis: Limitations of the '100 Relevant Events' rule

- In many real life conditions with clinical samples it can be **extremely difficult** or even **impossible** to collect 100 Relevant Events to stick to the rules.

- Multicolor flow cytometry can be of great help in these cases, since the more the colors used, the more focused is the analysis of the rare population, thus making statistically acceptable also the collection of **FEWER** Relevant Events.

- Statistical studies on **clinical samples** analyzed by multicolor flow analyses have established that **20-30 Relevant Events** are enough to classify a rare population as **DETECTABLE**, and at least **50 Events** can make such population **QUANTIFIABLE**.

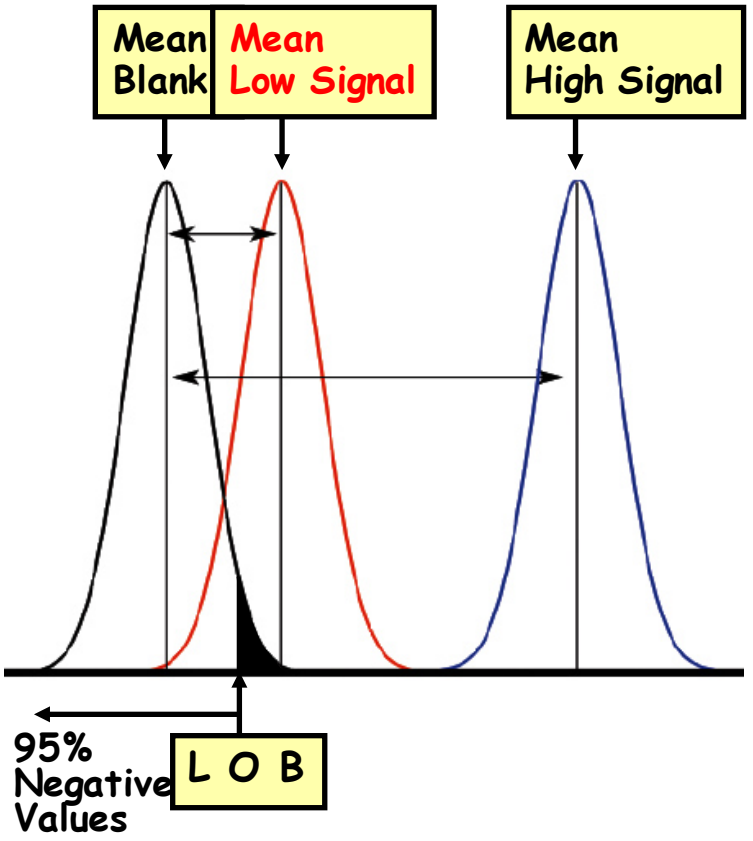
- Under **experimental controlled conditions** these limits may further be reduced to **~10 Events** (*just in vitro!*), provided very large amounts of events are captured (see: *Sutherland DR. Cytometry Part B (Clinical Cytometry) 2012; 82B: 195-208*).

- Such studies have introduced another important concept: the establishment of the lower limits of Detection (**LOD**) and of Quantitation (**LOQ**), that are not fixed values, but **VARY ACCORDING TO THE TOTAL NUMBER OF COLLECTED EVENTS**.

- *Lower* **Limit of Blank (LLOB):**
  The highest signal in the absence of the measurand.
  (Mean Blank + SD x 1.65).
  95% of negative values are below this limit.

- *Lower* **Limit of Detection (LLOD):**
  (Mean Blank + SDlow x 1.65).
  95% of negative values are above this limit.
  5% false negatives and
  5% false positives are assumed

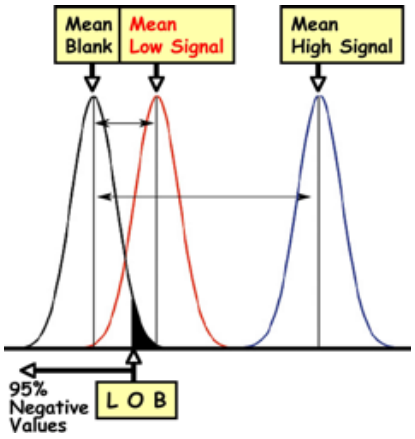
- *Lower* **Limit of Quantitation (LLOQ):**
  The lowest level of measurand that can be reliably quantitated at a predefined criterion for precision and accuracy (clinical utility value). Never lower than LOD.



Such concepts derive from clinical chemistry and can be directly applied to Flow Cytometry for INTENSITY measurements only.

Wood B. ICSH/ICCS. Cytometry Part B 2013; 84B: 315-323.

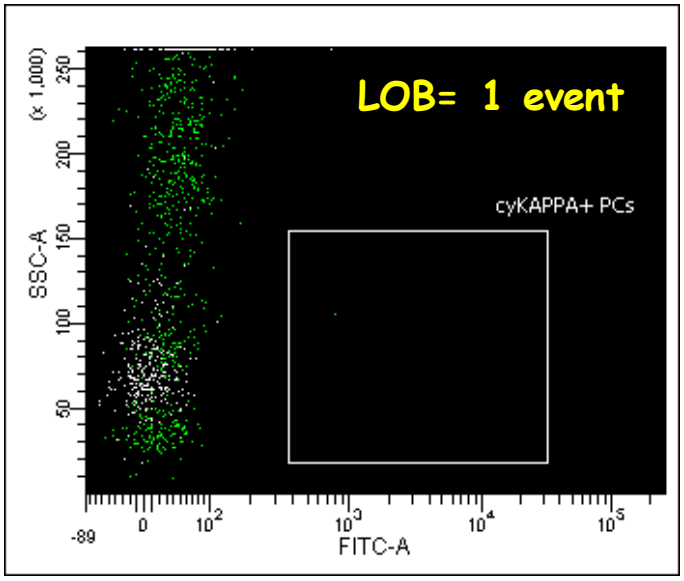
The LOD and LOQ Concepts Applied to Rare Event Analysis in FCM Studies

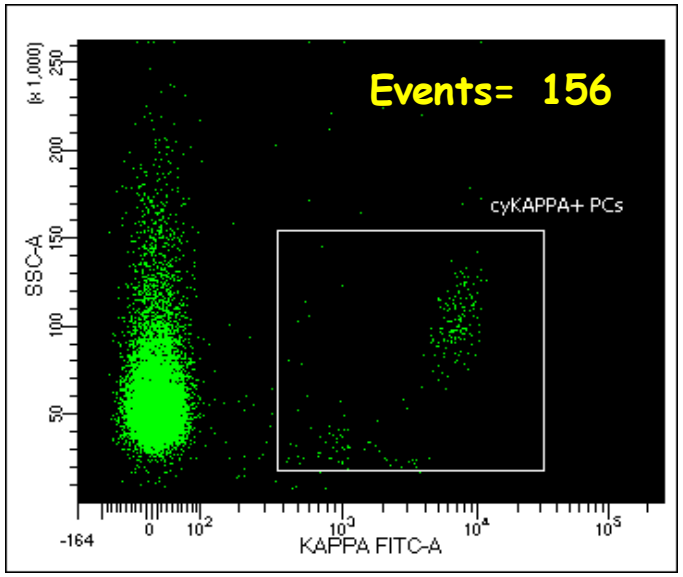


In FCM Rare Event studies, the **LOB / LOD / LOQ** concepts must be appropriately **translated** to be used with **EVENTS:**

LOB = Background events in the final window (**Best** = **0**, or to be subtracted)
LOD = **> 30** Events should be collected to say "**Detected**"
LOQ = **> 50** Events should be collected to **Quantitate** the population

[30 and 50 events derive from statistical estimations with Multicolor FCM]





Rare Event Studies by FCM: LLOD & LLOQ Change According to the Cell Denominator (total acquired cells) and to the Cell Numerator (relevant events)

Cell Denominators

Total Number of Acquired Cells ('Clean' cell events)	LLOD % ≥ **30** Events	LLOQ % ≥ **50** Events
100,000	0.03	0.05
200,000	0.015	0.025
500,000	0.006	0.01
1,000,000	0.003	0.005
2,000,000	0.0015	0.0025
3,000,000	~ 0.001	~ 0.0017
5,000,000	~ 0.0006	~ 0.001

Cell Numerators

The specific LLOD and LLOQ for the total amount of acquired cells should be reported.

Arroz M. Cytometry Part B 2016; 90B: 31-39.

Estimated LLOQ and LOQ According to the Total Number of Cells Acquired:

A Matter of Politics

Total number of gated cells acquired	Quantitative assay using LLOQ (>50 PNH cells) (%)	Qualitative assay using LOD (>20 PNH cells) (%)
10,000	0.5	0.2
20,000	0.25	0.1
30,000	0.17	0.066
40,000	0.125	0.05
50,000	0.1	0.04
100,000	0.05	0.02
200,000	0.025	0.01
300,000	0.017	0.007
400,000	0.0125	0.005
500,000	0.01	0.004
1,000,000	0.005	0.002

Denominators

Numerators

Establishing the LOD value **is a matter of politics** (not science).
Some researchers have adopted **20** events as the LOD (instead of 30).

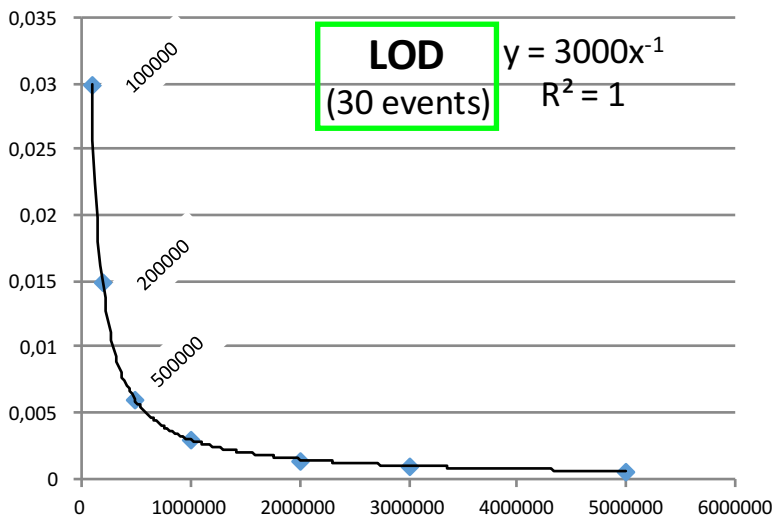
- Illingworth A. Cytometry Part B (Clinical Cytometry) 2018; 94B: 49 – 66.
- Sanoja-Flores L. Blood Cancer Journal 2018; 8: 117

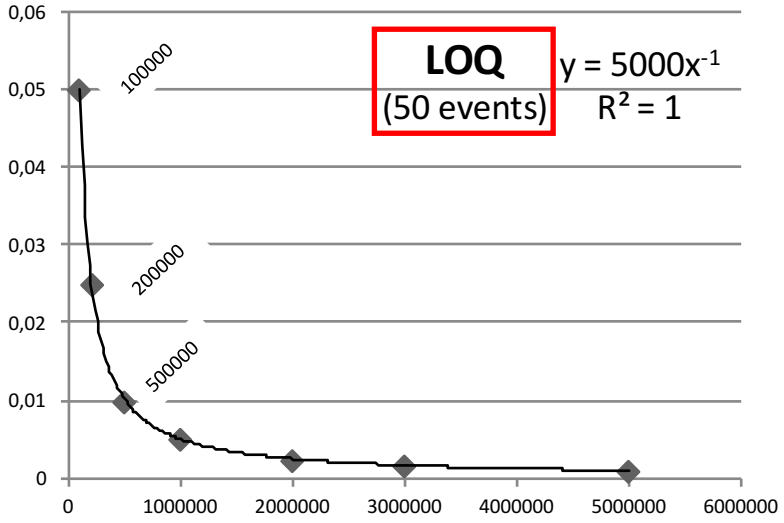
Estimated LOQ and LOQ According to the Total Number of Cells Acquired

To be practical:

LOD = **3000** *(or 2000, if you prefer)* / **Total n.** of Clean Events

LOQ = **5000** / **Total n.** of Clean Events





*Or **2000**, if you prefer, for 20 events*

$$\frac{3000}{\text{Total Number of Clean Events}} = \text{LOD}$$

$$\frac{5000}{\text{Total Number of Clean Events}} = \text{LOQ}$$

Limits of Detection / Quantification

Small numbers of POSITIVE target cells lead to errors in the results

	Number of target cells detected		
Total number of cells analysed	2 cells	20 Cells (C.I. 95%)	50 Cells (C.I. 95%)
100 K	0.002 (0.00024 - 0.0072)	0.02 (0.012 - 0.031)	0.05 (0.037 - 0.066)
200 K	0.001 (0.00012 - 0.0036)	0.01 (0.0061 - 0.015)	0.025 (0.019 - 0.033)
500 K	0.0004 (0.000048 - 0.0014)	0.004 (0.0024 - 0.0062)	0.01 (0.0074 - 0.013)
1 million	0.0002 (0.000024 - 0.00072)	0.002 (0.0012 - 0.0031)	0.005 (0.0037 - 0.0066)
2 million	0.0001 (0.000012 - 0.00036)	0.001 (0.00061 - 0.0015)	0.0025 (0.0019 - 0.0033)
5 million	0.00004 (0.0000048 - 0.00014)	0.0004 (0.00024 - 0.00062)	0.001 (0.00074 - 0.0013)

- **Using 20 cells as target, 95% Confidence Intervals may mean from 12 to 31 cells**
- **Using 50 cells as target, 95% C.I. from 37 to 65 cells**

- 95% C.I. for 2 Cells→ 1.5 Log error; for 20 Cells→ 0.4 Log error; for 50 Cells→ 0.25 Log error
- According to Poisson's statistics, the overall magnitude of the sample loses importance, while **the chosen number of Positive target events really makes the difference.**

Courtesy of Andy Rawstron, 2015, modified.

Rare Event Studies by FCM: **LOD** & **LOQ** Change According to the Cell Denominator (*total acquired cells*) and to the Cell Numerator (*relevant events*)

- The introduction of LOD and LOQ concepts in rare event analysis has been a remarkable advancement to ensure robust and reliable measurements of rare events.

- The major practical consequence is that if a sufficient amount of clean events is not collected, the sensitivity of a measurement **CANNOT BE HIGHER** than the one indicated in the table.

- Another important consequence is the **need to specify the LOD and LOQ in each measurement and report**, since they change according to your luck *(say how many total and relevant cell events you were able to collect in that experiment).*

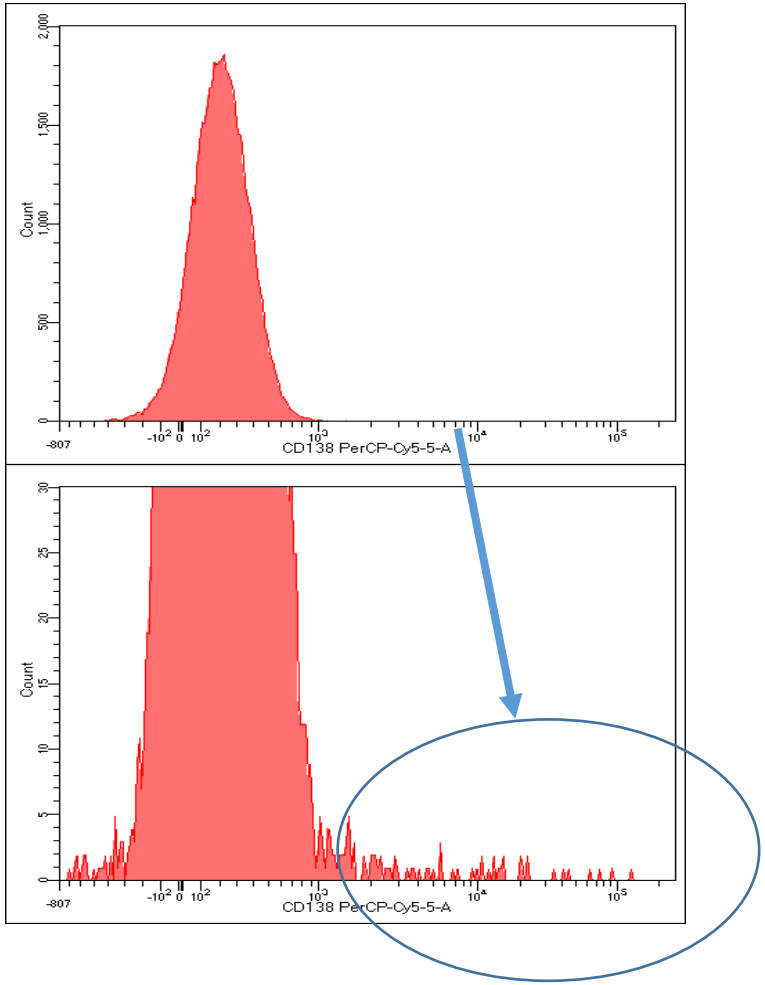
- Example.1: 15 events/5,000,000 = 0.0003% (LOD ≥ 0.0006%) → **Not Detectable (<LOD)**

- Example.2: 35 events/2,000,000 = 0.0017% (LOD ≥ 0.0015%, LOQ ≥ 0.0025) → **Detectable but Not Quantifiable (>LOD, <LOQ)**

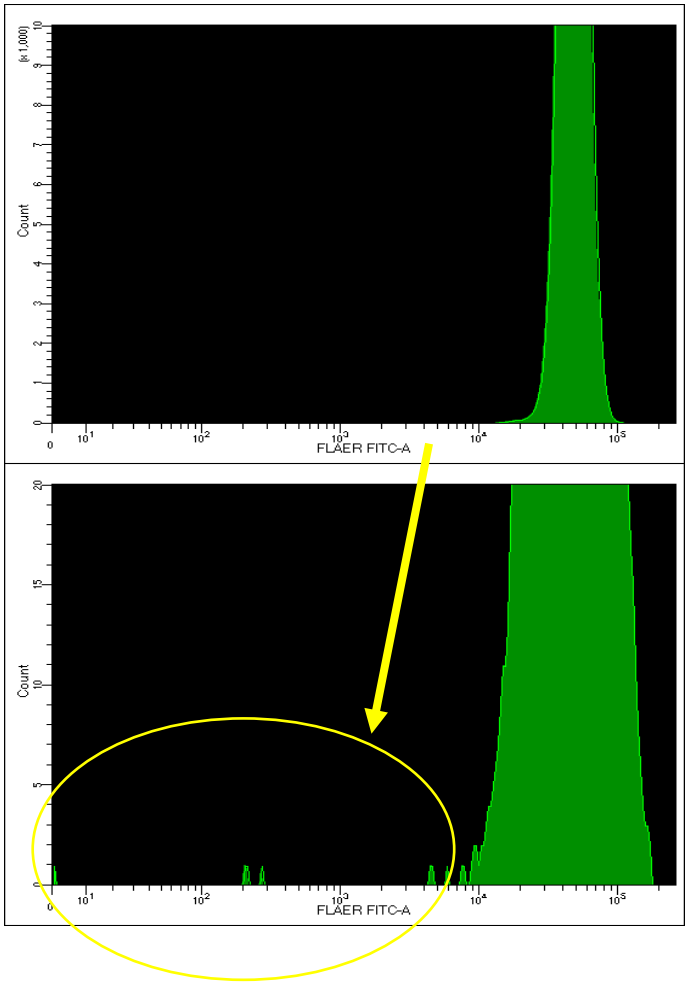
- Example.3: 1,000 events/2,000,000 = 0.05% (LOQ ≥ 0.0025%) → **Quantifiable (>LOQ)**
  And only in this case you are allowed to calculate and report a PERCENT VALUE

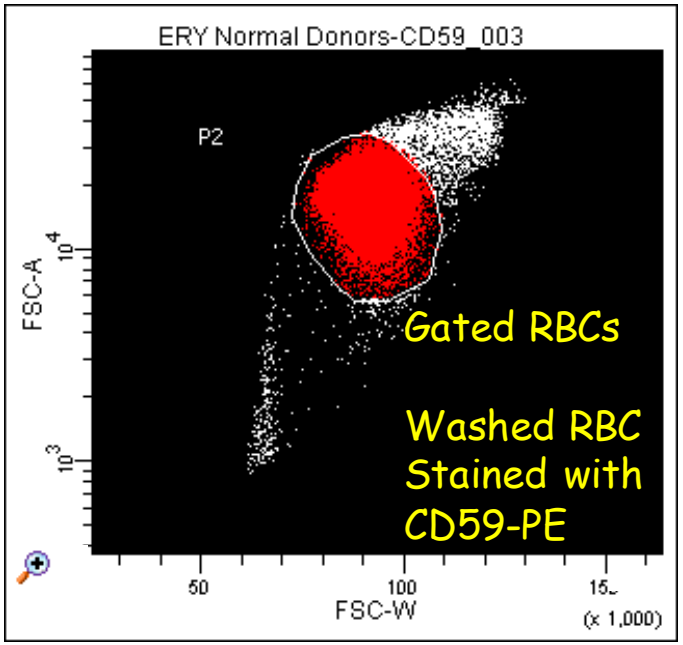
The Two Faces of the Background

The '**POSITIVE**' Background Events



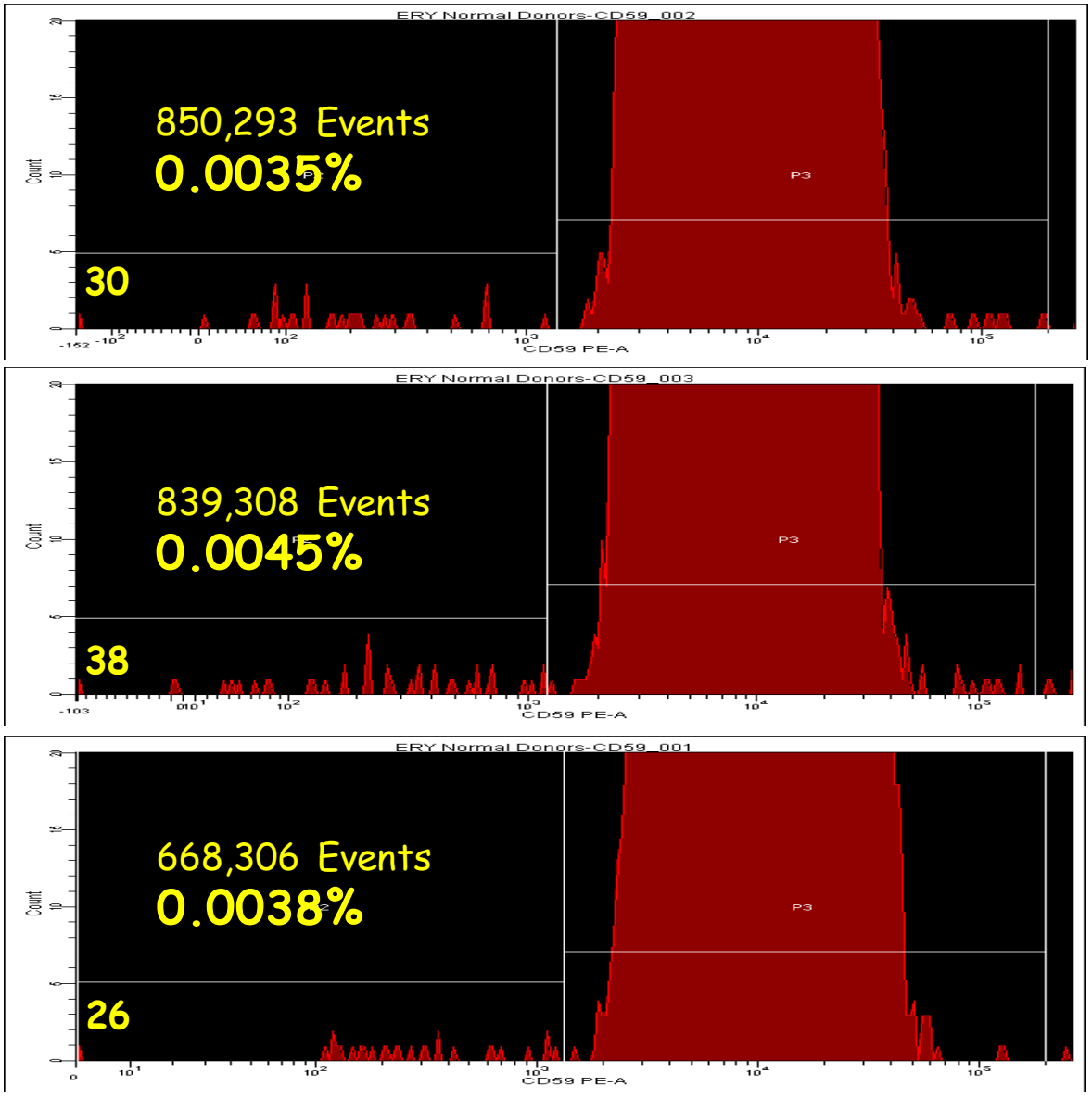
The '**NEGATIVE**' Background Events

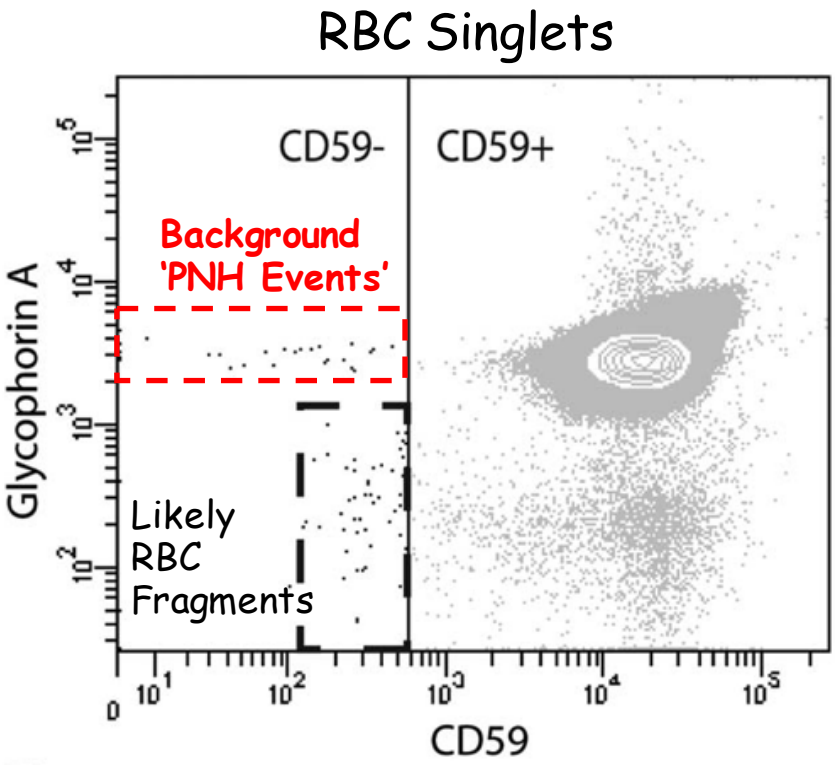


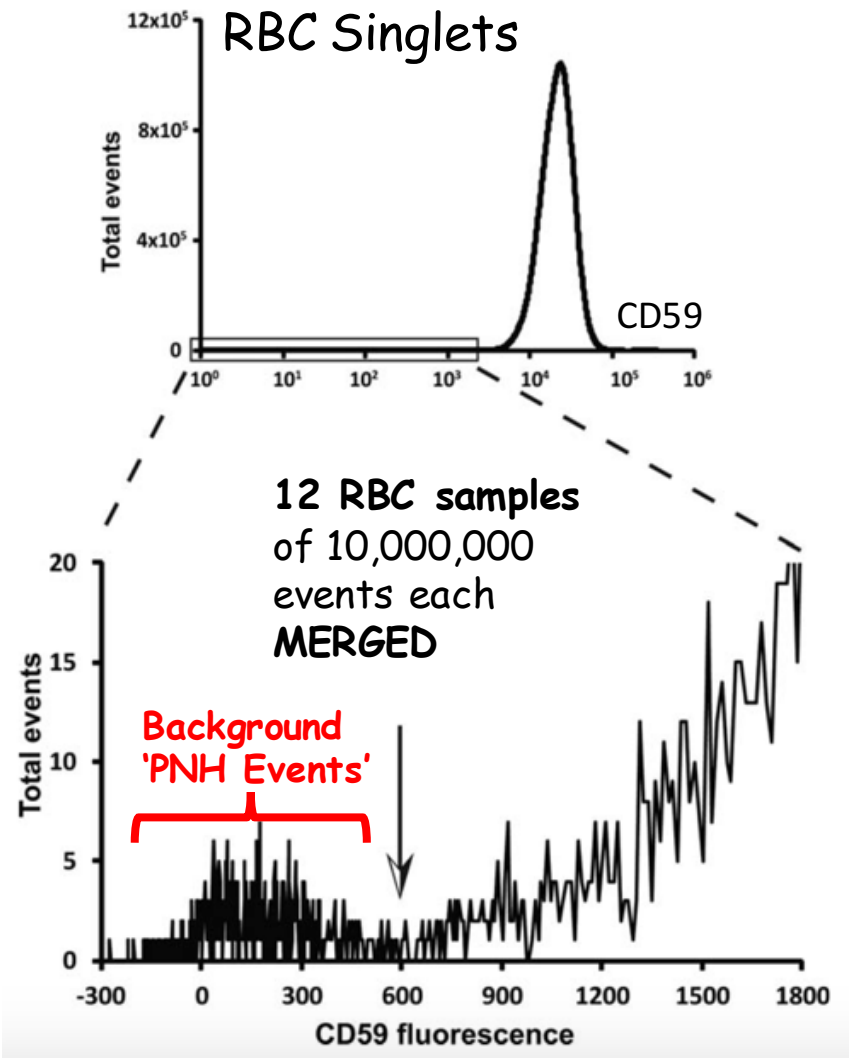


Analysis of **NEGATIVE** Background

'PNH Red Blood Cell Events' in normal subjects (CD59-PE).







The median background **'PNH' RBC events in normals** are from 3 to 5 per 100,000, with a range from 0 to 17 or 95% C.I. ~ 0.002-0.007% .
LLOQ is ~ 0.005% (RBC Gated with CD235a FITC)

Liew M. Cytometry Part B (Clinical Cytometry) 2015; 88B: 261–269.

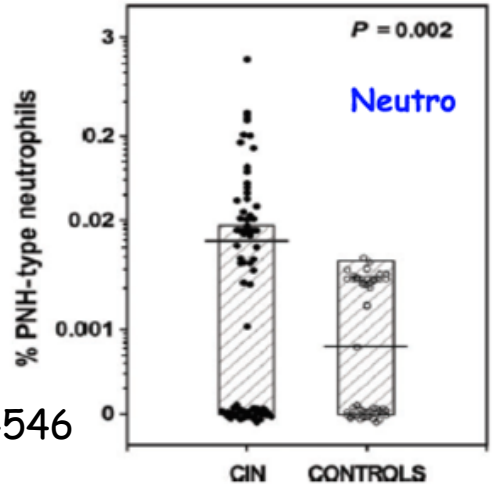
High-Sensitivity PNH assay (White Cells)

Establishing the NEGATIVE Background of 'PNH WBC Events' in Normal Samples

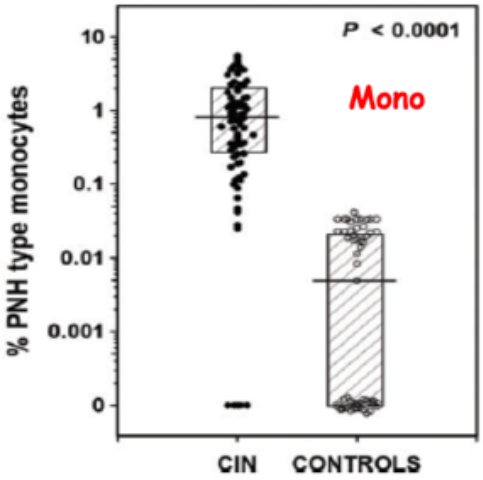
Gatti A.
Eur J Haematol 2017; 99: 27-35.

	Normal Donors	
	6 Color Mean% (range)	**2 Color Mean%** (range)
Background **NEUTROPHIL** 'PNH CLONE SPACE' %	**0.0008** (0 - 0.0029)	**0.001** (0 - 0.0036)
Background **MONOCYTE** 'PNH CLONE SPACE' %	**0.0208** (0 - 0.13)	**0.254** (0.025 - 0.719)

Damianaki A.
Eur J Haematol 2016; 97: 538-546



Control Group: PNH events
median 0.006% (0.0-0.0071%)



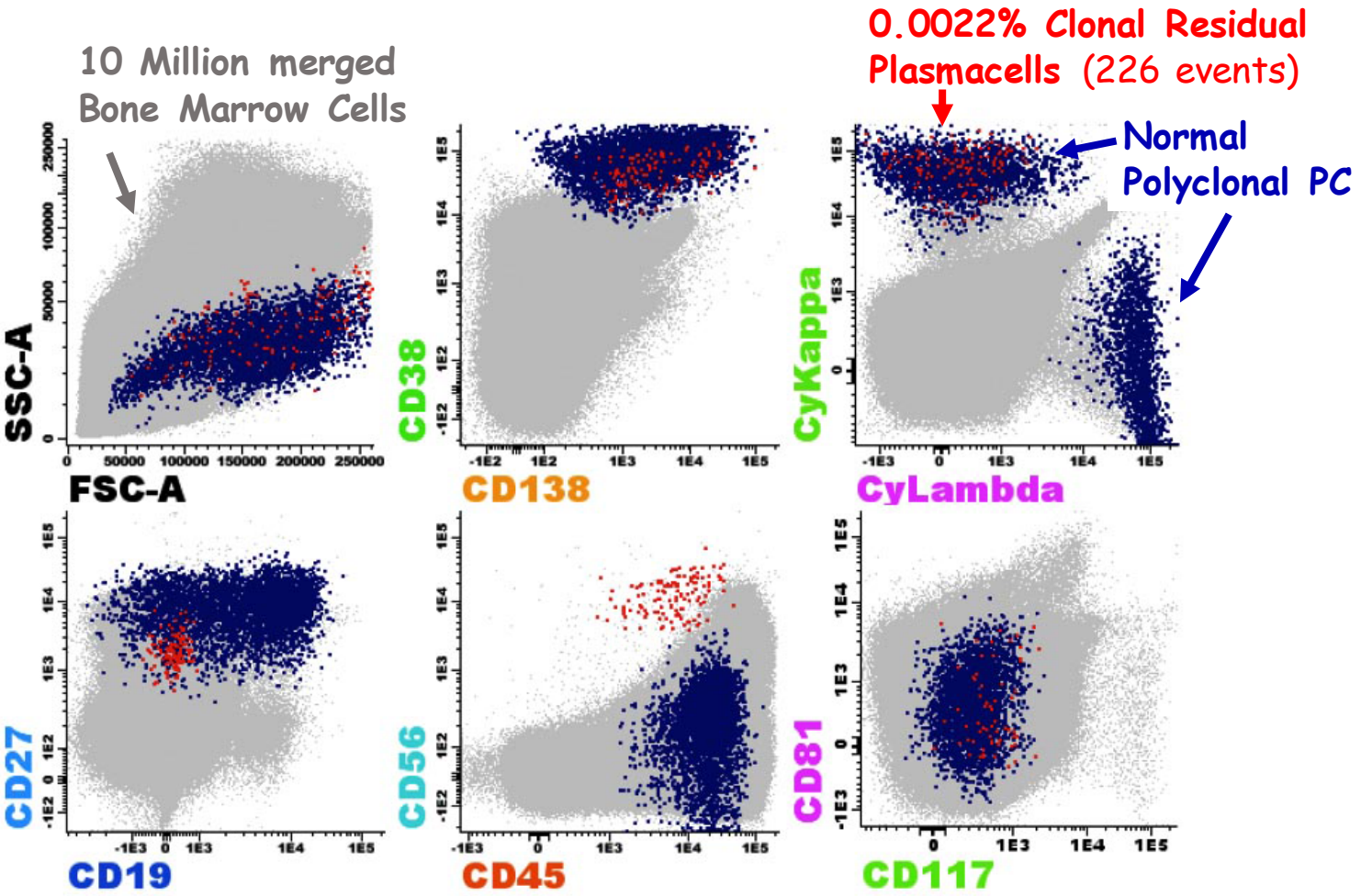
Control Group: PNH events
median 0.0212 % (0.0-0.0414%)

Deeper and Deeper: **Next Generation Multicolor Flow Cytometry** to Reach 10^{-5}

Using the INFINICYT ™ Analysis Software

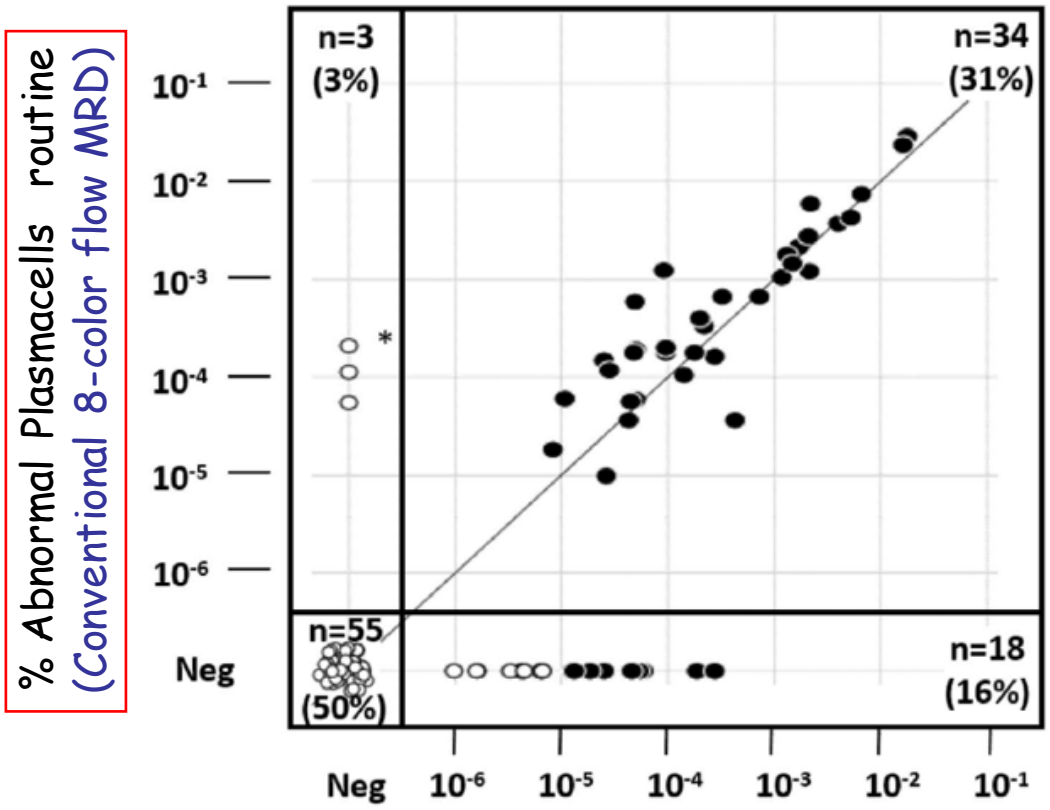
Infinicyt allows the electronic merging of multiple multicolor tubes with common backbone markers, to generate bulky cell datafiles and to focus on rare cell detection and enumeration.

In this example 5+5 Million Bone Marrow cells are merged, with 226 residual clonal plasmacells: Minimal Residual Disease analysis at 10^{-5} sensitivity.



Flores-Montero J. Cytometry Part B 2016; 90B: 61-72.

Next Generation Flow Minimal Residual Disease vs Conventional 8-color Flow MRD



Next Generation Flow Cytometric analysis is a brand new technique that proved superior to conventional multicolor FCM in high-sensitivity Minimal Residual Disease (MRD) studies.

In this example, a number of 'conventional FCM-negative' myeloma MRD cases were found to be positive by Next Generation FCM analysis at 10^{-4} to 10^{-5} sensitivity levels.

* Proven polyclonal by CyIg staining

Flores-Montero J.
Leukemia 2017; 31(10): 2094-2103

Next-Generation Flow-MRD is now considered equivalent to ASO-qPCR and NGS in Myeloma

	Allele-specific oligonucleotide qPCR	Multicolor Flow Cytometry	VDJ sequencing
Applicability	60–70%	Nearly 100%	≥90%
Need for baseline sample	Yes, requires production of patient-specific probes	Not required; abnormal plasma cells can be identified in any sample by their distinct immunophenotypic pattern *vs* normal plasma cells	Baseline samples required for identification of the dominant clonotype; alternatively, a stored sample from a time point with detectable disease can be used to define baseline status
Sample requirements	<1 million cells	>5 million cells	<1 million cells; higher numbers improve sensitivity
Sample processing	Can be delayed; can use both fresh and stored samples	Needs assessment within 24–48 h; requires a fresh sample	Can be delayed; can use both fresh and stored samples
Sample quality control	Not possible. Additional studies required	Immediate with global bone marrow cell analysis	Not possible. Additional studies required
Sensitivity	≥1 in 10^5	≥1 in10^5	≥1 in 10^5
Information regarding sample composition	No further information available	Detailed information available on leucocyte subsets and their relative distribution	Information about immunoglobulin gene repertoire of B cells in the studied patient samples
Turnaround and complexity	Labour intensive; requires the development of patient-specific primers/probes; can take several days	Can be done in a few hours; automated software available	Can take several days for turnaround; requires intense bioinformatics support. Use of local laboratories could speed up this limitation
Standardisation	Has been done for other diseases (EuroMRD), can be done for myeloma as well	Standardised by the EuroFlow consortium	In process
Availability	Wide*	Most hospitals with four-colour flow cytometry. Eight or more-colour flow cytometry requires more experienced centres/laboratories. Many laboratories have adopted the EuroFlow laboratory protocols and use the EuroFlow MRD tubes	So far limited to one company/platform

New International Myeloma Working Group (IMWG) Response Criteria

Kumar S. Lancet Oncology 2016; Aug (17): e328-e346

MRD In Clinical Trials – EMA & FDA Recommendations

- EMA myeloma: "It is recommended to use two different methods within the same trial".
- FDA: "The sensitivity of the MRD assay should be at least 10-fold below the clinical decision-making threshold (the definition of MRD). For example, if MRD positive or negative is defined as detection of greater or less than 1x10-5 cells, respectively, then the assay should be optimized and validated to have an analytical sensitivity of at least 1x10-6".

Courtesy of Andy Rawstron

MRD ANALYSIS:

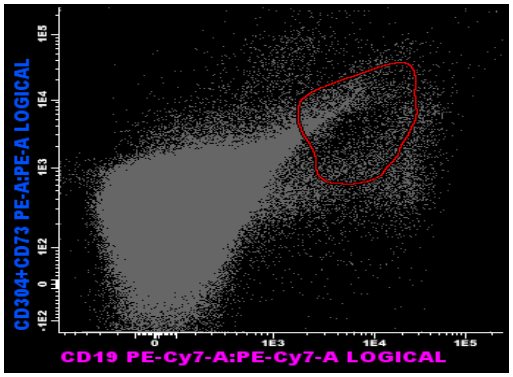
Highly dependent from MoAb Mixtures.

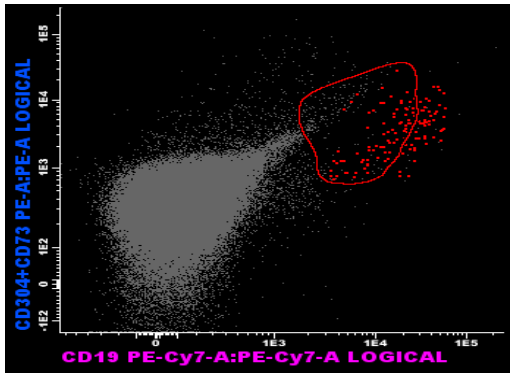
Highly dependent from sample matrix (i.e. blood vs Bone Marrow)

and

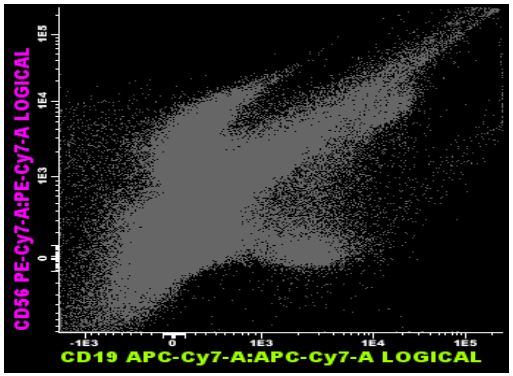
Strongly DISEASE-SPECIFIC

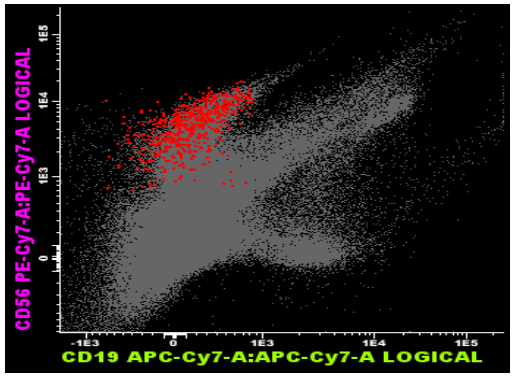
(*one MRD strategy does NOT fit all !*)



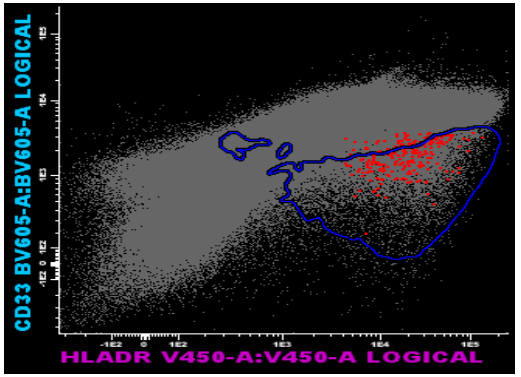


B-ALL
1,473,210 Clean Events
MRD = 697 (0.047%)



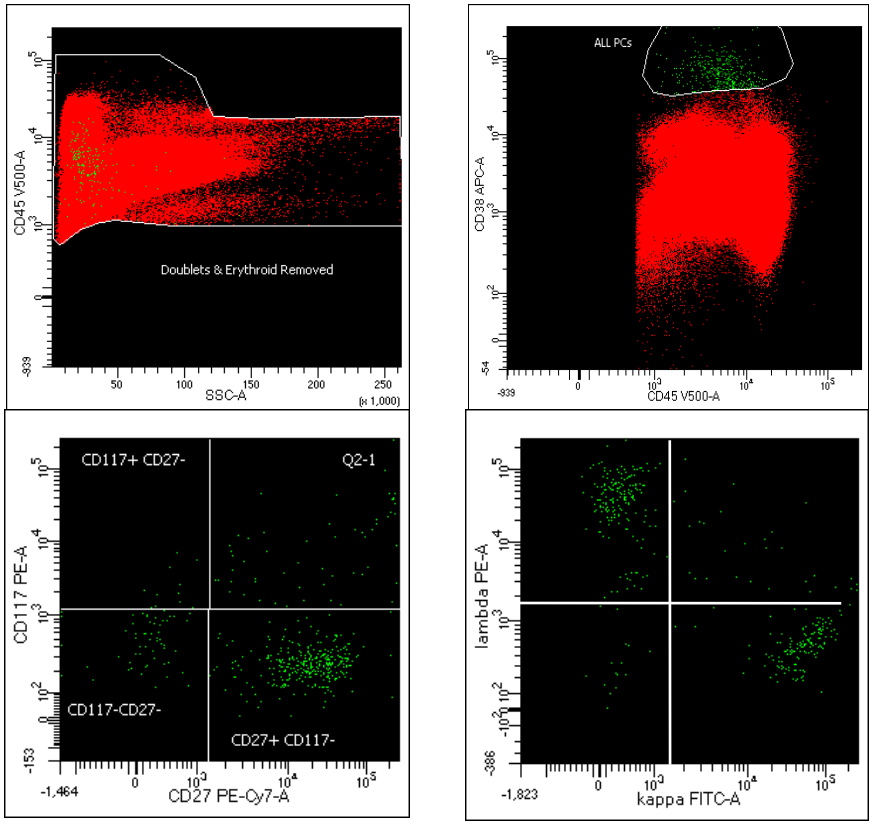


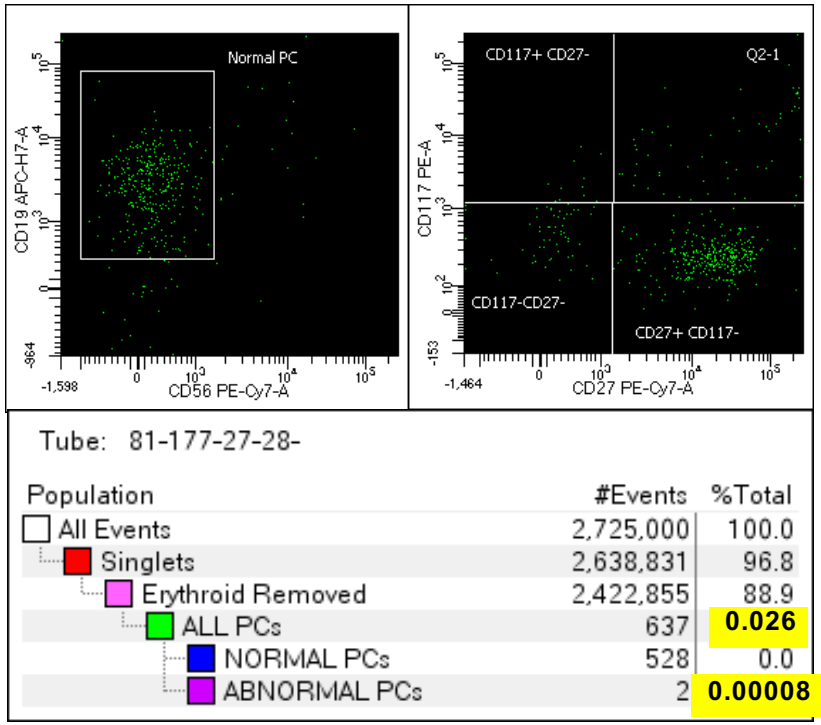
MM
1,537,104 Clean Events
MRD = 1,390 (0.090%)



AML
789,450 Clean Events
MRD = 83 (0.010%)

Rare Event Analysis - Examples: **MRD-Negative Myeloma Case in follow-up**





Tube: 81-177-27-28-

Population	#Events	%Total
All Events	2,725,000	100.0
Singlets	2,638,831	96.8
Erythroid Removed	2,422,855	88.9
ALL PCs	637	**0.026**
NORMAL PCs	528	0.0
ABNORMAL PCs	2	**0.00008**

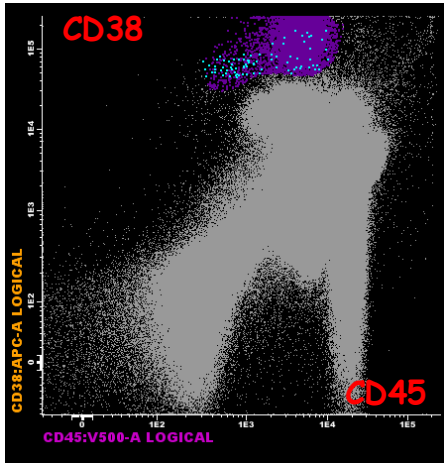
- Kappa FITC/Lambda PE/CD138 PerCP Cy5.5/CD27 PE Cy7 CD38 APC/CD19 APC H7/CD28 H450/CD45 H500

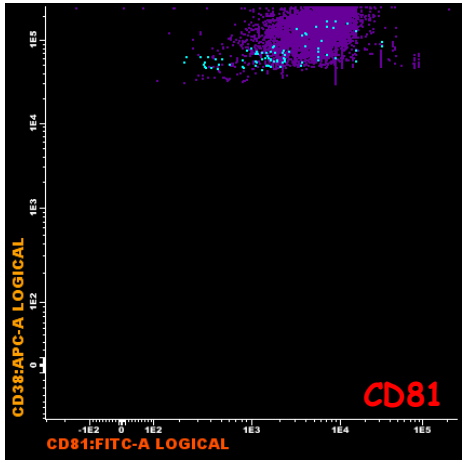
Conventional cytometric analysis lacks the same stringent rules applied in Next Generation Cytometry

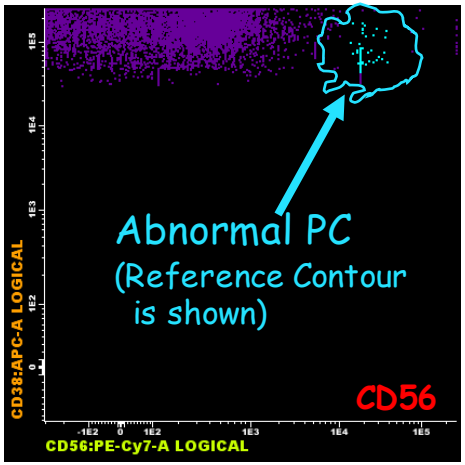
- 1 Tube, 8 Color, Conventional Analysis with FACSDiva
- 2.422 Mill. BM cells acquired (Doublets and erythroid removed)
- LOD = 0.0012%

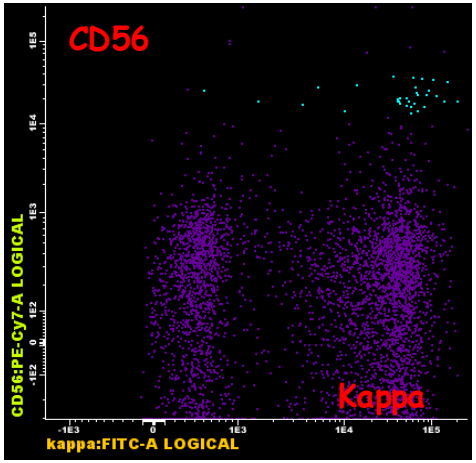
- Only 2 Abnormal PCs collected (0.00008%, <LOD)
- Conclusion: NOT Detectable, MRD Negative

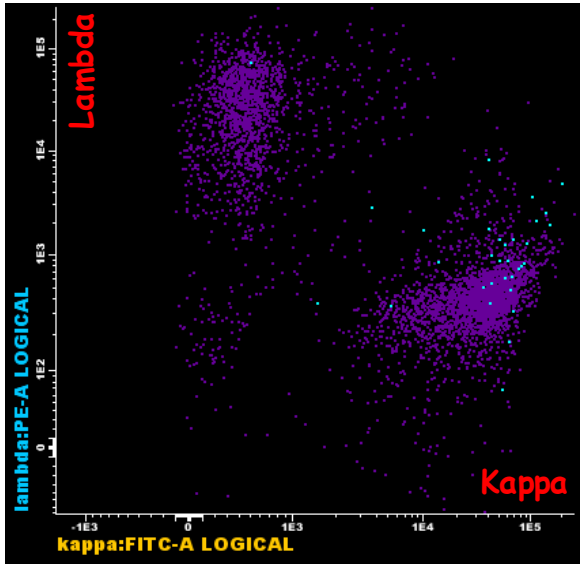
Rare Event Analysis - Examples: **MRD-Positive Myeloma by Next Generation FCM**









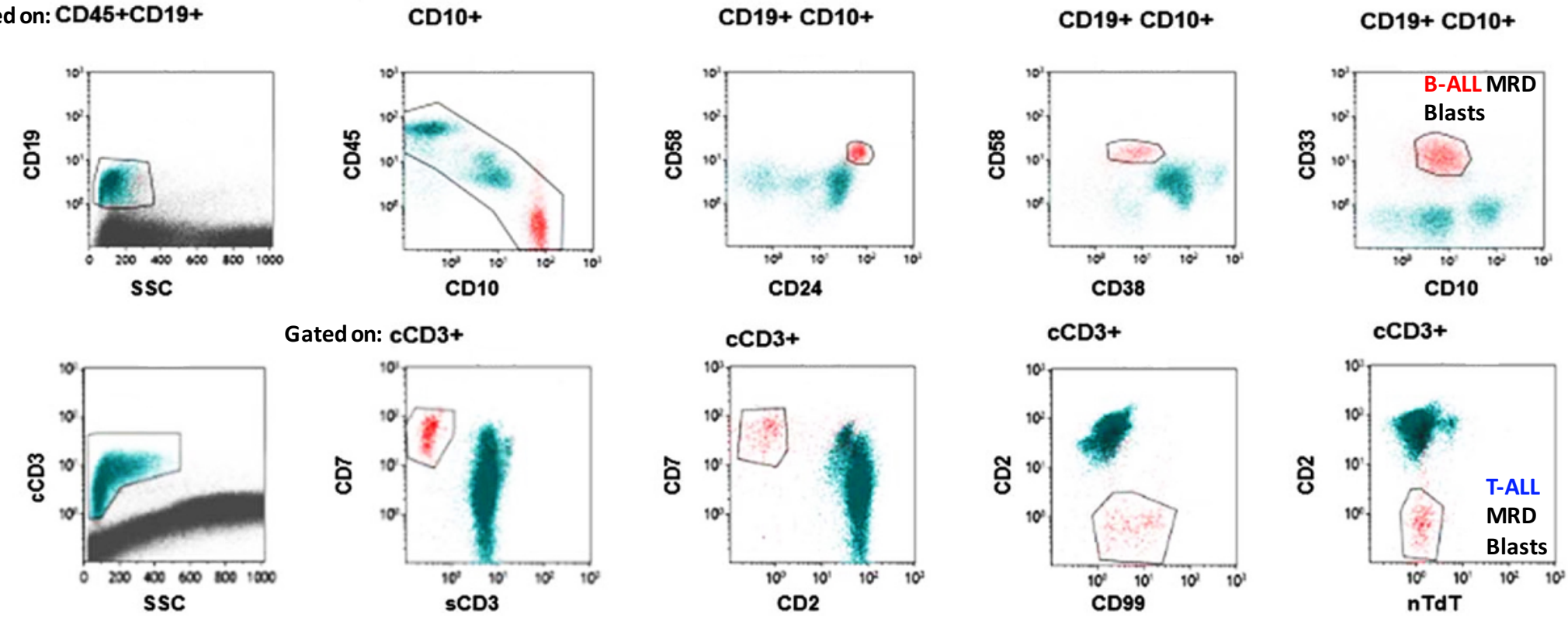


- 2 EuroFlow 8-Color tubes merged, Infinicyt Software
- 2.677 Million BM cells acquired (Doublets and Erythroid removed)
- LOD = 0.0012% LOQ = 0.002 %

- 81 Abnormal PCs collected (0.003%, >LOD and > LOQ)
- Rare events are detectable and quantifiable: **MRD Positive**

Rare Event Analysis - Examples: **Acute Lymphocytic Leukemia MRD Studies**

B-ALL MRD: CD10/CD19/CD24/CD58/CD33/CD38/CD45. MRD Blasts: 450/700,000 = 0.064%

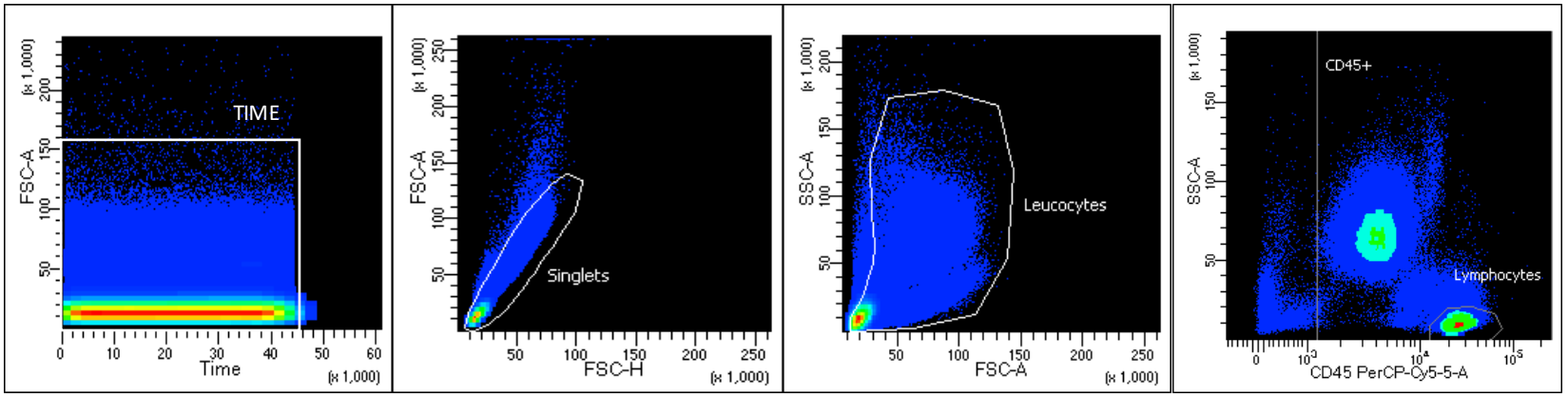


T-ALL MRD: nTdT/cCD3/sCD3/CD2/CD7/CD99/CD45. MRD Blasts: 430/200,000 = 0.21%

Fossat C. Cytometry Part B (Clinical Cytometry) 2015; 88B: 21-29.

Rare Event Analysis - Examples: **High-Resolution PNH Detection and Quantitation**

1-Tube 6-Color (White Cells Only): FLAER Alexa 488 / CD24 PE/ CD45 PerCP-Cy5.5/ CD15 APC/ CD64 PE-Cy7/ CD14 APC-Cy7



Small PNH clones of clinical relevance (i.e. >0.1% can be better detected in Granulocytes rather than in Monocytes, due to the lower frequency of the latter.

Tube: FLAER /24/15/45/14/64_001

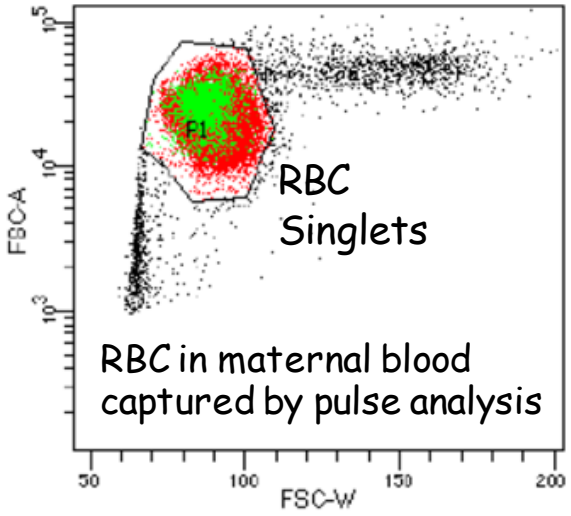
Population	#Events
All Events	3,425,000
TIME	749,435
Singlets	339,882
Leucocytes CD45+	286,735
Lymphocytes	45,958
GRANULOCYTES	99,207
CD24- FLAER-	68
CD24-	0
MONOCYTES	10,980
CD14- FLAER-	36
CD14-	300

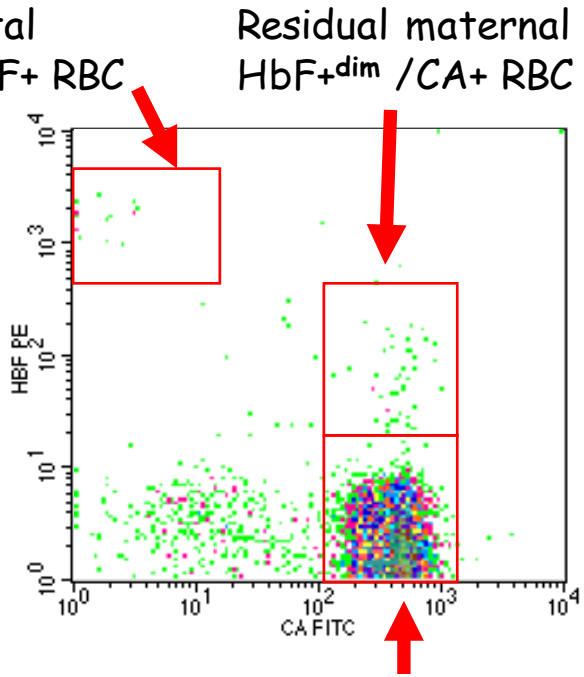
Granulocyte PNH Clone: 68/99,207 = **0.068%** - Monocyte PNH Clone: 36/10,980 = **0.32%**

It is often difficult to collect a sufficient number of Monocytes to perform a true High-Sensitivity PNH Analysis.

Rare Event Analysis - Examples: **Analysis of Feto-Maternal Hemorrhage (FMH)**

- Fetal RBC may spill into maternal circulation, sometimes for a number of obstetrical problems.
- Fetal RBC intensely express intracellular fetal Hb (**HbF**), whereas mature maternal RBC express Carbonic Anhydrase (**CA**).
- In every healthy adult a few **residual HbF+^{dim} RBC** can be detected, which also express CA. Such cells can be greatly increased in carriers of abnormal hemoglobins.





FMH = 69 /471,904 = 0.014%
(>LOQ, Quantifiable)

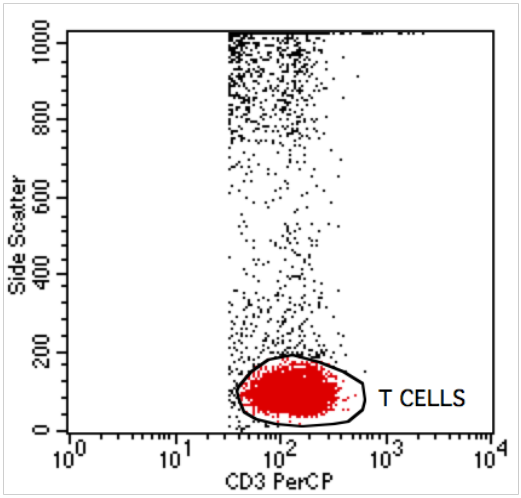
The quantitation threshold of HbF+ /CA- cells is > 0.007% (LOQ).

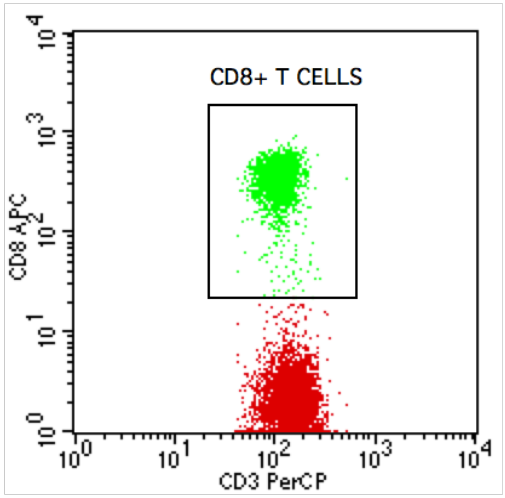
The amount of fetal blood volume spilled into the maternal circulation can be quantified (to guide Anti-Rh/D Prophylaxis).

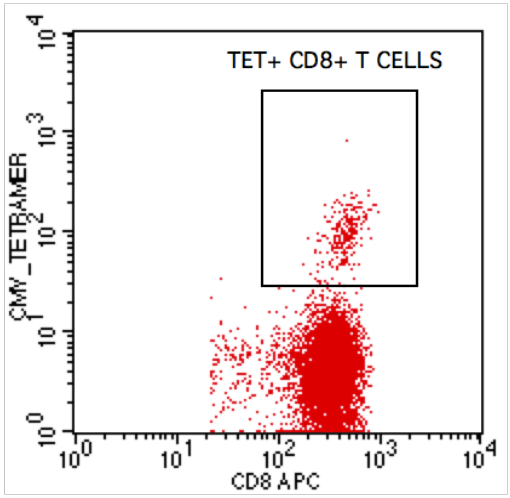
Population	#Events
All Events	500,000
Time	480,028
RBC Singlets	471,904
Hb F+	69
Hb F+ CA+	578
x	56,801
CA+	414,456

Rare Event Analysis - Examples: **Detection of Antigen-Specific CD8+T Cells**

HLA-Multimers or Dextramers are charged with antigenic peptides and conjugated with fluorochromes to bind antigen-specific CD8+ T Cells to quantitate the Antigen-Specific T Cell response.

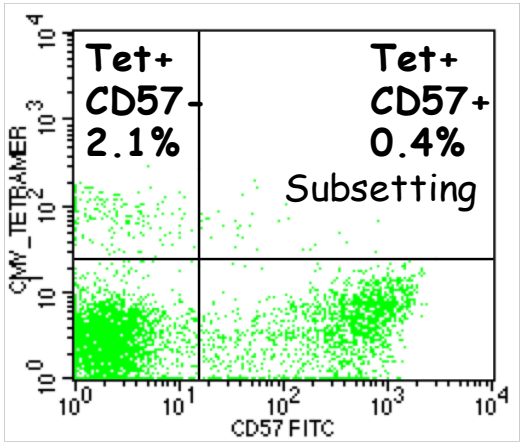


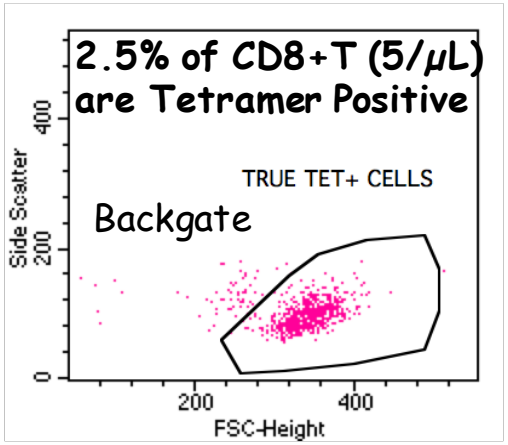




A HLA Class I Tetramer is mounted with the CMV peptide **NLVPMVATV** and conjugated with PE.

The complex binds to CMV-experienced CD8+ T Cells of patients with the HLA-A*0201 aplotype.



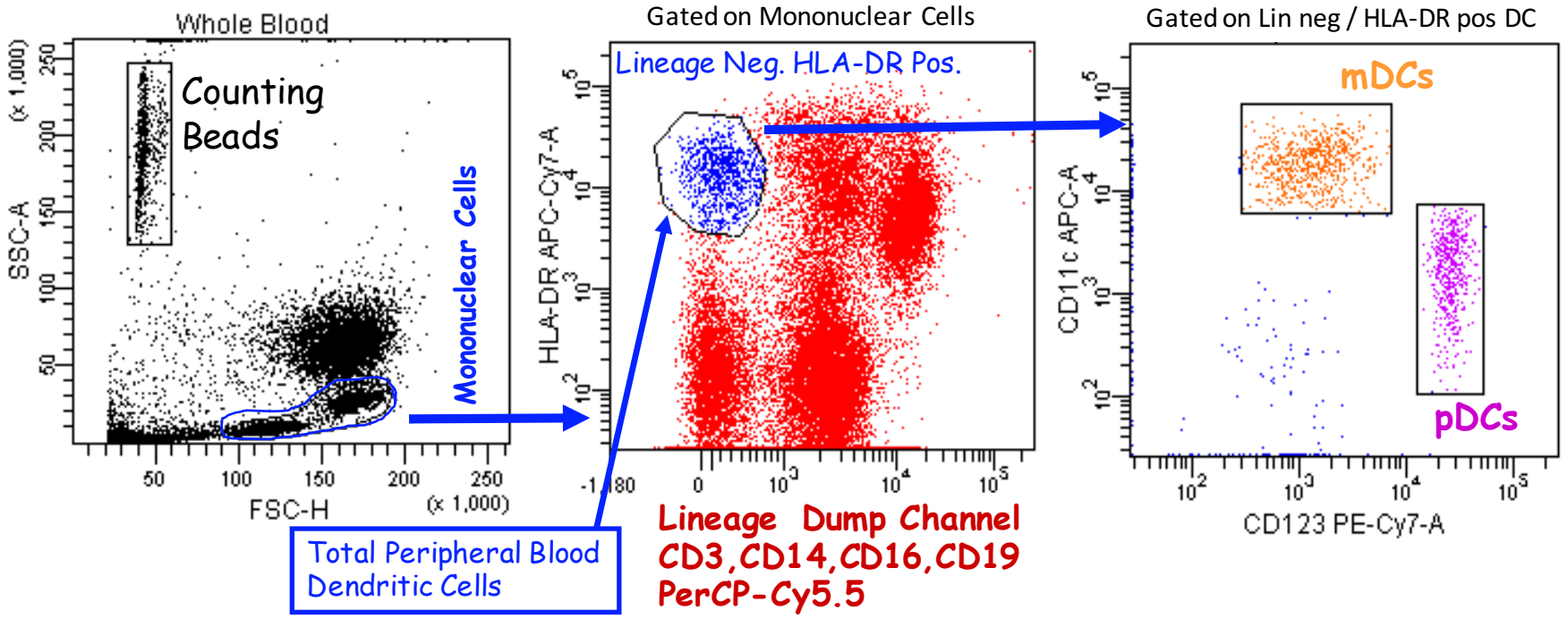


HLA-Multimer FCM technique is used to assess the occurrence and the quantitatiton of a T Cell antigen-specific response in infection and vaccination studies.

Antigen-specific CD8+ T Cells are usually within the 'Rare Event' frequency (FSC/SSC backgating is always recommended).
Further subsetting of Tet+ Cells is possible (i.e. CD57)

Rare Event Analysis - Examples: **m- and p-Dendritic Cells in Peripheral Blood**

Basic Gating Panel: Lineage Dump Channel PerCP-Cy5.5/ CD123 PE-Cy7/ CD11c APC/ HLA-DR APC-Cy7



Both DC subsets are further characterized by activation and functional markers in various combinations:

- CD80 *or* CD86 *or* IL10 *or* IL12 *or* IFNα **FITC** *and*
- CD83 *or* CD40 *or* TNFα *or* IL4 *or* IL6 **PE**

Courtesy of Prof. Silvia Della Bella

Rare Event Analysis – **A Special Case: Low Level Leucocyte Count (LLLC)**
Residual White Cell Detection in Leucoreduced Blood Products

- Leucocytes are removed by on-line filtration from red blood cell and platelet concentrates, to prevent a number of untoward clinical effects.
- Leucoreducing filters remove very efficiently white cells, down to 0-3 residual WBC/μL, i.e. <500,000 rWBC per blood component bag.
- Monitoring the effectiveness of leucoreduction is part of the quality control of blood bank activities.
- rWBC are detected by staining the DNA of their nuclei by Propidium Iodide, which ensures a very bright signal and a predictable positioning of the rWBC cluster.
- rWBC detection is a special case of Rare Event Analysis: relevant cells are simply quantified if present in a predefined volume unit of the checked sample using counting beads. rWBC may be virtually undetectable in many cases.
- Just one color is enough, and no cell denominator is applicable.

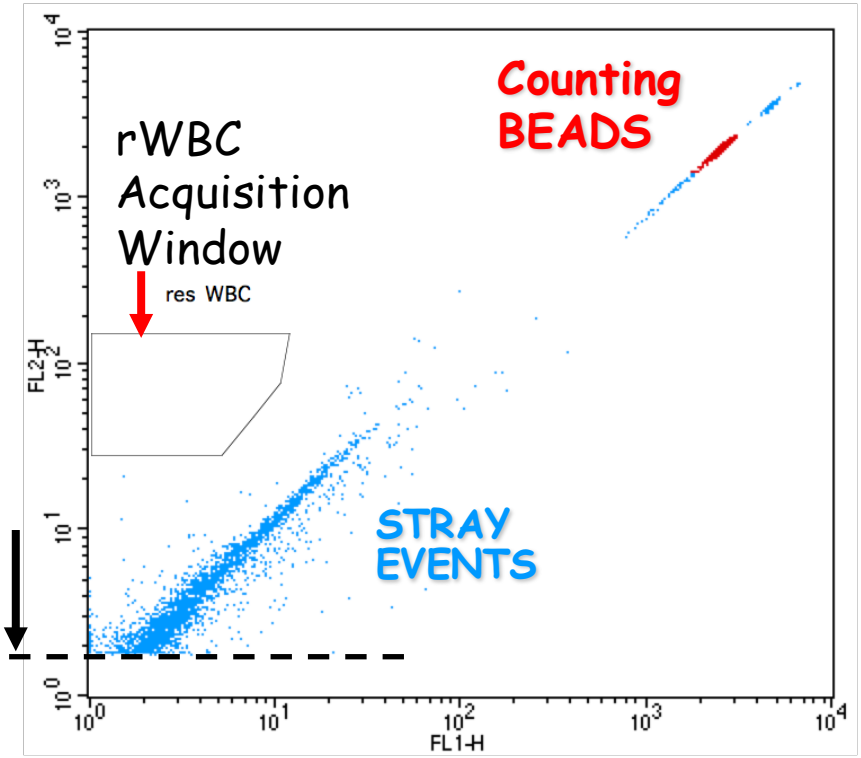
Rare Event Analysis – **A Special Case: LLLC**

Residual White Cell Detection in Leucoreduced Blood Products

50-100 µL
ANY
BLOOD
PRODUCT

50-100 µL
Flow-Count
BEADS
(Or TruCOUNT
Tube)

500 µL
Propidium/
Detergent/
RNAse
STAINING
MEDIUM



**REDUCED FL2
THRESHOLD**
(to show stray events)

**NEGATIVE CONTROL:
Medium + Counting
Beads.**

Ensure the virtual
absence of events
in the rWBC acquisition
window.

Stray events are generated by RBC or PLT membranes destroyed by the detergent. They are unavoidable, but the 'Angle of Dangle' diagonal pattern keeps them far from the rWBC acquisition window on FL2.

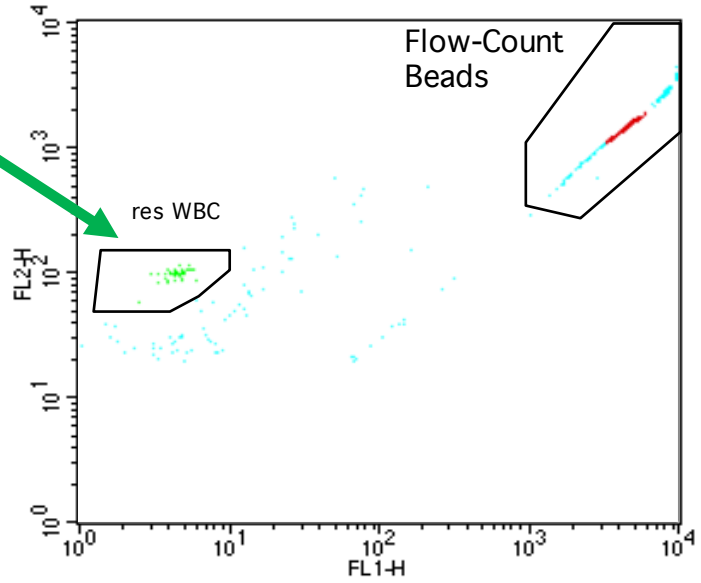
Rare Event Analysis – **A Special Case: LLC**

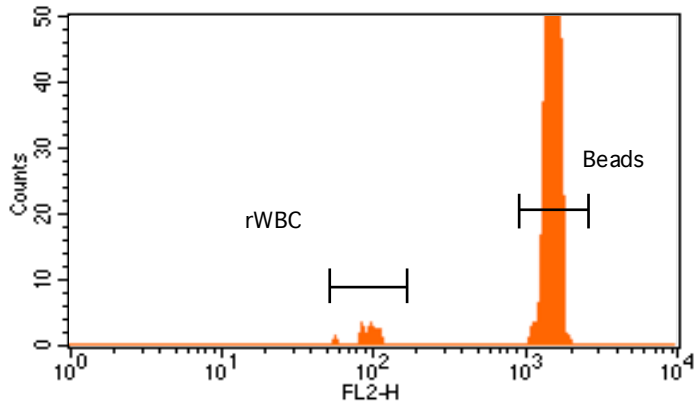
Residual White Cell Detection in Leucoreduced Blood Products

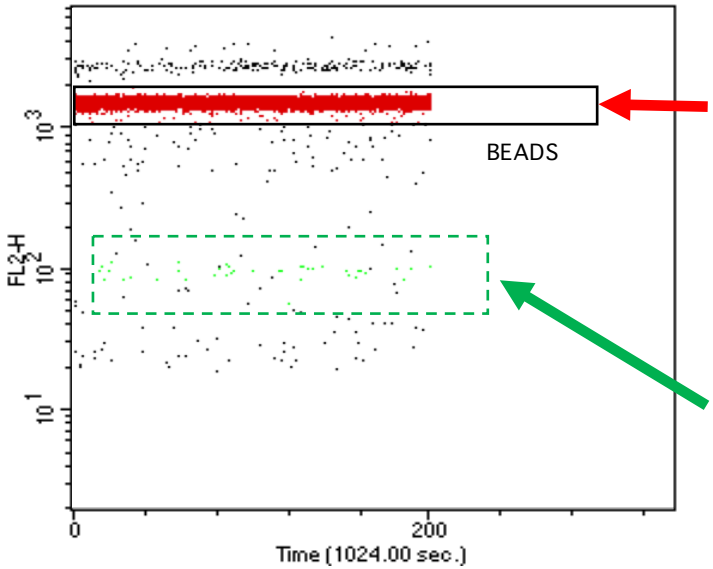
Residual WBC

FL2 PMT is set to read rWBC at 100 Units.

(Propidium generates a reproducible, stoichiometric DNA staining)







The regularity of the long acquisition run is monitored by the counting beads. Acquisition of 15,000 – 20,000 beads indicate an adequate sampling.

The rare rWBC events appear at an irregular rate.

FLOWCOUNT Bead Lot = 1026 beads /µL

Marker	Events	Mean
All	22884	1496.19
rWBC	39	101.50
Beads	20845	1499.88

Bead suspension concentration is provided by the manufacturer

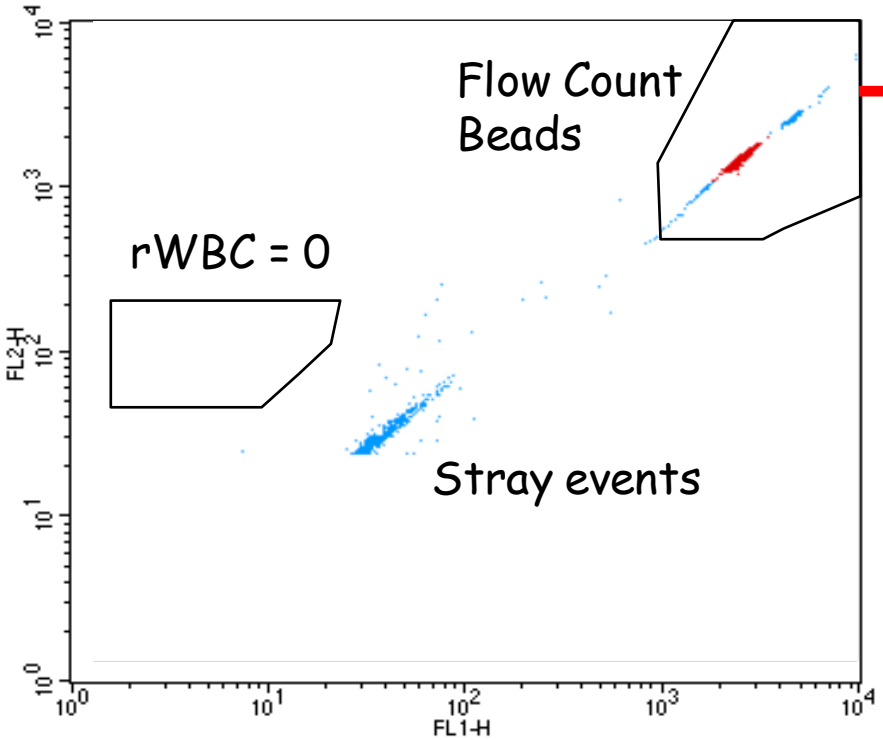
rWBC = 39 / 20845 * 1026 = 1.91/µL

- The ordinary bead-based absolute count method is applied.
- Less than 3 rWBC/µL indicates an effective leucoreduction.

Rare Event Analysis – **The last Frontier: LLLC**

Residual White Cell Detection in Leucoreduced Blood Products: Managing the ZERO Events

If 'Rare Events' are virtually absent, you may spend at the cytometer the rest of your life, being unable to collect the recommended 30, 50 or 100 relevant events !



Counting Beads are added to samples at **~1000/ µL**, so that acquiring 1000 beads means that around 1 **µL** of the original sample has been analyzed.

- **ZERO Events** is the last frontier of Rare Event Analysis, where a different approach is needed.
- In case **NO rWBC** events at all are collectable and **no cell denominator** is available, you may decide to stop the acquisition when a predefined amount of sample has been analyzed (i.e. stop when 20,000 beads are collected, that is when ~20µL of the original sample have been analyzed).

One Last Discussion Point: **RARE** event vs **SCARCE** event Analysis

The Case of FCM Analysis of **CSF** for Hematologic Malignancies

- In CerebroSpinal Fluid (**CSF**) FCM analysis we are faced with very low numbers of cell events admixed to usually low-grade background.

- This condition is technically more similar to Low Level Leucocyte Count rather than to MRD analysis.

- The point is the capture of a meaningful number of relevant cell events (i.e. malignant cells and accompanying leucocytes).

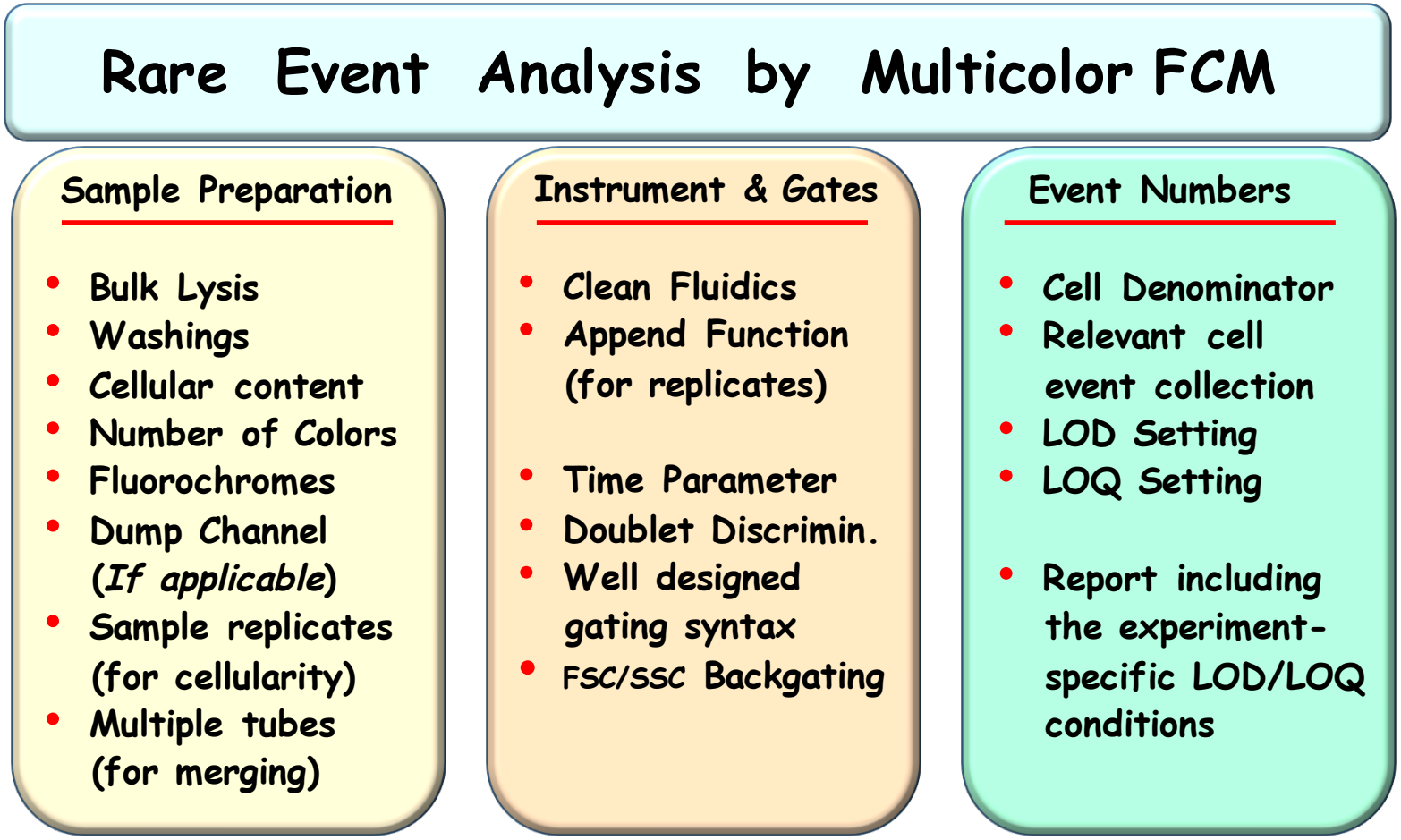
- The lowest number of relevant events to ensure a clear diagnosis of '**positive**' or '**negative**' CSF is the real problem, and still a matter of debate (i.e. from 5 to 30 events in the literature).

The Road from Routine to Next Generation Flow Cytometry

A Tool For MRD and High-Resolution FCM Analysis

Frequency of Relevant Cells	Total Cell Events to be Acquired	Analytical Context	Applications / Examples
10^{-2}	10,000	Routine FCM	ROUTINE PHENOTYPING CD34 ANALYSIS
10^{-3}	100,000	Rare Event Analysis by FCM	DENDRITIC CELLS FMH AML-MRD, 'MEASURABLE' MRD Ag-SPECIFIC T CELLS
10^{-4}	1,000,000	High Resolution FCM *(First Generation FCM)*	HI-RES PNH CLL-MRD B SUBSETS in RITUXIMAB CEC, DENDRITIC CELLS
10^{-5}	10,000,000	Next Generation FCM	ALL-MRD MYELOMA-MRD

Conclusion: The Three Technical Pillars of Rare Event Analysis by Multicolor FCM



With the full control of such technical prerequisites, any clinical and experimental Rare Event Analysis become reliable and can be undertaken with confidence.