Emoglobinuria Parossistica Notturna

Focus Clinico e Update Diagnostico

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ANALISI DI EPN AD ALTA RISOLUZIONE

Fino a Dove ci Possiamo Spingere ?

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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
10^{-5}	10,000,000	Next Generation FCM	ALL-MRD MYELOMA-MRD

Why to Detect and Monitor Minor (<1%) and Very Small (<0.1%) PNH Clones

- Small PNH clones can be detected in up to 50-70% of patients with Aplastic Anaemia (AA) or MDS, usually without haemolysis.
- In adult BM failures, the presence of a small PNH clone may predict response to immunosuppressive therapy (*according to SOME studies*).
- Care must be taken to discriminate TRULY PNH-negative patients from subjects with very small PNH clones (i.e. < 0.1%).
- AA patients with small PNH clones are at higher risk of developing clinically overt PNH after high-dose immunosuppression.
- Small clones can grow over time (6-7%) and should be monitored.

- Killick SB. Br J Haematol 2016; 172: 187–207.
- Zhao X. Ann Hematol 2015; 94: 1105-1110.
- Kulagin A. Br J Haematol 2013; 164: 546-554.
- Pu JJ. Eur J Haematol 2011; 87: 37-45.

How to Manage PNH High-Resolution Analysis (i.e. <0.1% Clones)

- High-Resolution assays are usually not needed for the diagnosis of classical, clinically overt PNH.

- High-Resolution FCM analysis must be used for the detection of **small PNH** populations (<0.1%) in patients with bone marrow failure disorders.

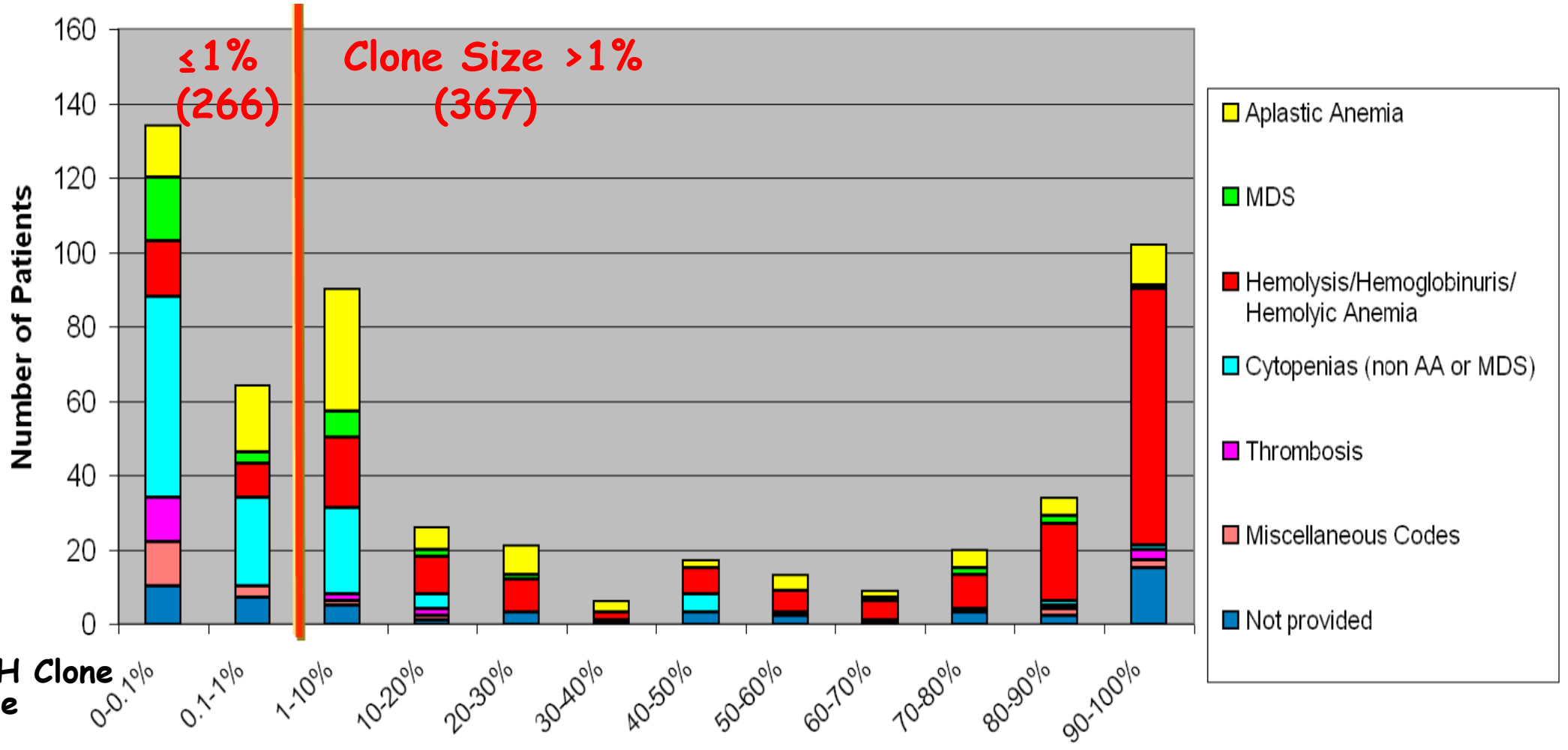
- High-Resolution PNH analysis must **follow the requirements of rare event assays** (i.e. at the same level as B-CLL MRD studies).

- High-Resolution PNH analysis can be easily performed on **Red Cells** and **Neutrophils** (except in severe aplastic conditions), but it is difficult to perform on **Monocytes, due to the lower frequency of such cells**.

- Great care should be especially taken with putative small 'Type II' PNH clones (often artefactual) and with Monocytes.

High-Sensitivity FCM for Accurate PNH Diagnosis and Monitoring

42% of PNH+ Samples Show a Clone of <1%



Size of Neutrophil PNH clones in 633 PNH+ patients/10,236 with a reason for testing.

Illingworth A. Cytometry Part B (Clinical Cytometry) 2018; 94B: 49 – 66.

Technical Requirements for the Accurate Detection of Small PNH Clones

- Ensure a **careful cleaning** of the fluidic system.
- Ensure the **maximal specificity** of the staining protocol (*titration*).
- Prepare a washed cell-rich sample to collect a **high number of cell events**.
- Take care of **fluidic perturbations** during long acquisitions, using TIME.
- Set a well designed **gating syntax** aimed at eliminating non-specific events.
- **Event numbers** may be **more important** than the number of colors.
- Acquire the highest possible amount of **clean 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.
- Fluidic perturbations generate a lot of false signals.
- Excess fluorescence spillover generates a lot of false signals.
- Gating syntax has a great impact → Elimination of doublets, Gating out fluidic perturbations, 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>30**; **LOQ>50** events.
- Experimental conditions are VERY DIFFERENT from real life.

Validation of High-sensitivity PNH assay (White Cells)

Establish the Frequency of Background PNH WBC Events in Normal Samples

	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)

Table I. Frequency of background PNH-like events falling into the 'PNH Clone Spaces' for Neutrophils and monocytes, respectively evaluated in 25 normal subjects with the state-of-the art 6-color and with the simplified 2-color method. At least 50,000 Neutrophils (range 50,000-225,000) and 2,500 monocytes (range 2,500-22,500) were included in the analyses.

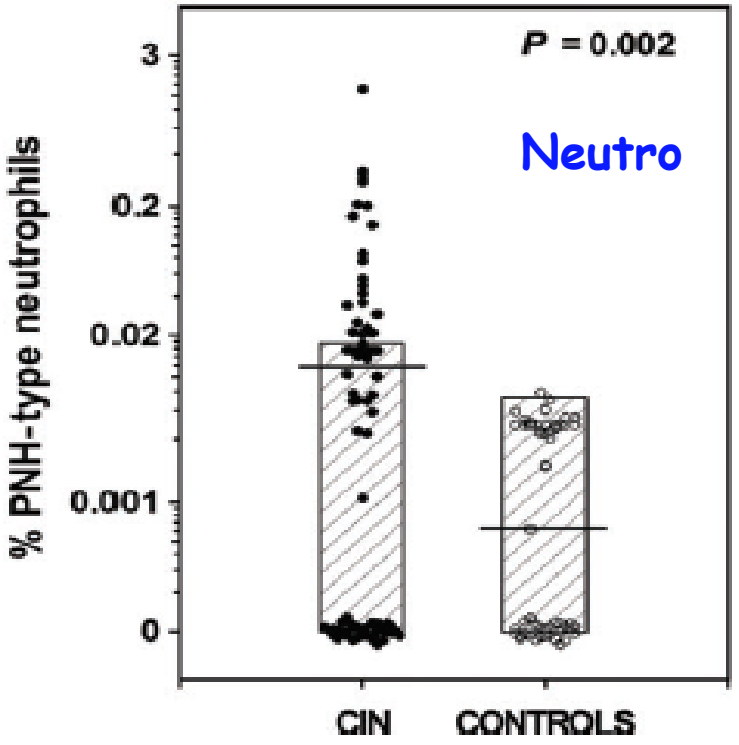
During the multicenter study all the 24 normal donor samples were correctly classified as 'PNH-Negative' by the six expert participants.

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

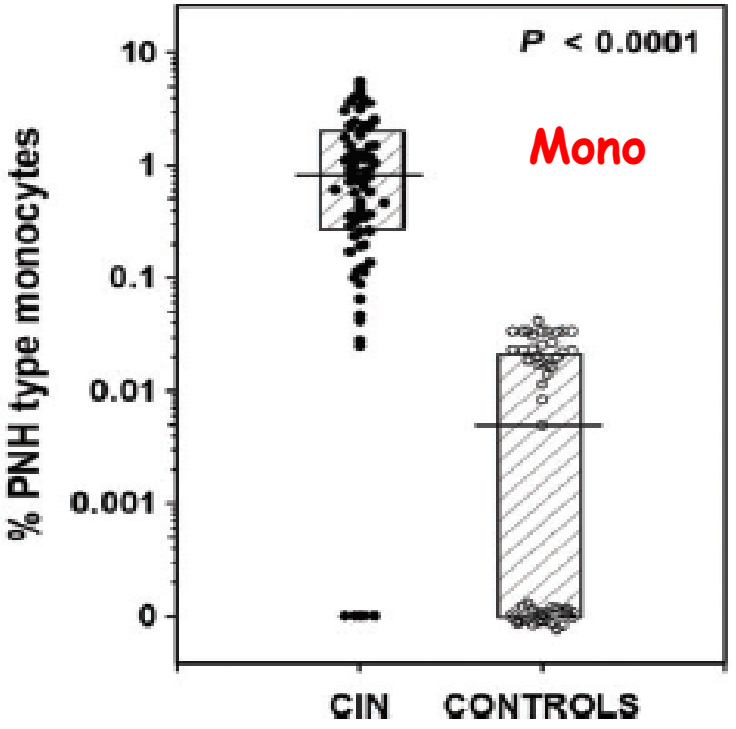
Validation of High-sensitivity PNH assay (White Cells)

Establish the Frequency of Background PNH WBC Events in Normal Samples

PNH-type cells in patients with Chronic Idiopathic Neutropenia (CIN)



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



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

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

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 Non–Paroxysmal Nocturnal Hemoglobinuria Samples

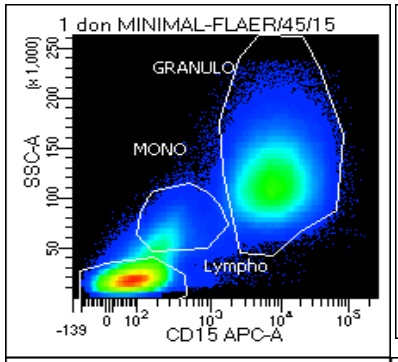
Cell Type/Parameter	Whole-Blood Staining	Washed Whole Blood	Lyse Before Stain
Granulocytes			
Mean No. of FLAER-negative cells among 250,000 granulocytes	2.16	1.7	1.66
No. + 5 SD	12.4	10.7	8
Limit of detection, %	0.0049	0.0043	0.0032
Monocytes			
Mean No. of FLAER-negative cells among 10,000 monocytes	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 is **higher with bulk lysis. Fixation is detrimental** to FLAER emission.

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

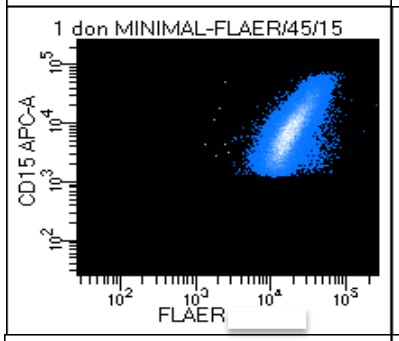
The Positive Effect on Background Events of Bulk Lysis vs the Conventional Stain-and-Lyse Procedure

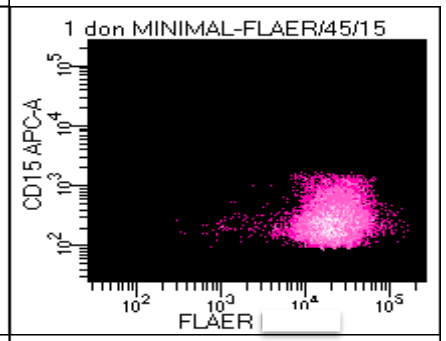
Stain-and-Lyse method

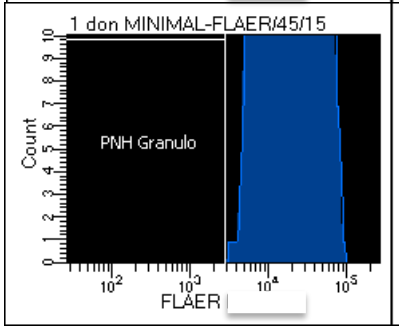


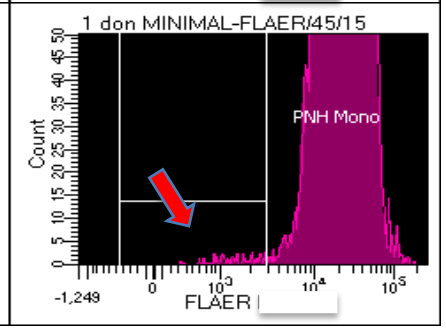
Tube: FLAER/15

Population	#Events	%Parent	%Total
All Events	391,494	####	100.0
Time	391,001	99.9	99.9
Singlets	388,070	99.3	99.1
ALL CELLS	349,942	90.2	89.4
GRANULO	130,208	37.2	33.3
PNH Granulo	7	0.0	0.0
MONO	23,387	6.7	6.0
Mono Refined	20,605	88.1	5.3
PNH Mono	67	0.3	0.0
Lympho	177,425	50.7	45.3









Same Sample,

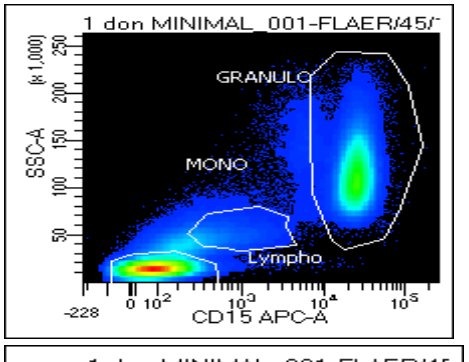
Same Setting,

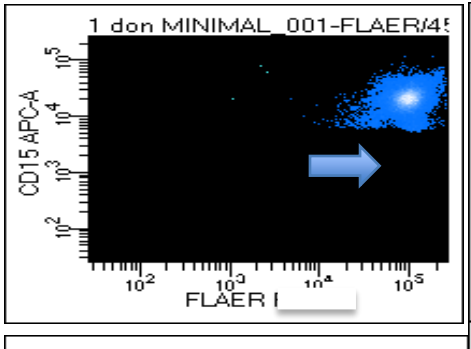
Same FLAER Amount!

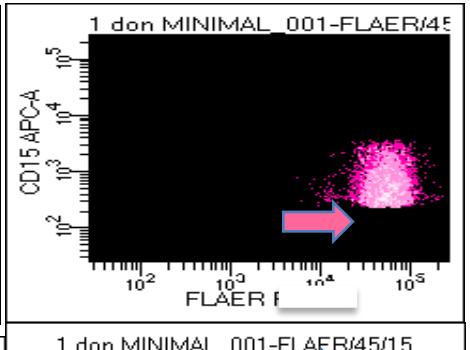
Bulk Lysis method

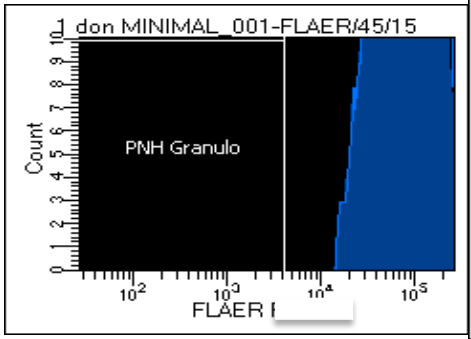
Tube: FLAER/15

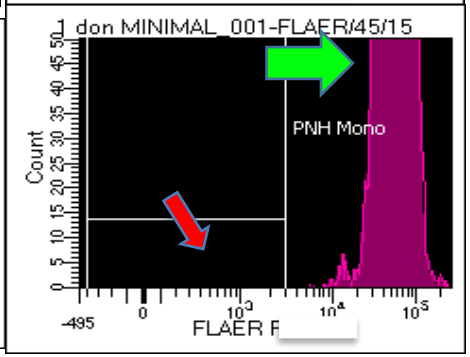
Population	#Events	%Parent	%Total
All Events	276,046	####	100.0
Time	248,329	90.0	90.0
Singlets	246,473	99.3	89.3
ALL CELLS	233,221	94.6	84.5
GRANULO	70,564	30.3	25.6
PNH Granulo	6	0.0	0.0
MONO	7,949	3.4	2.9
Mono Refined	7,686	96.7	2.8
PNH Mono	0	0.0	0.0
Lympho	135,690	58.2	49.2



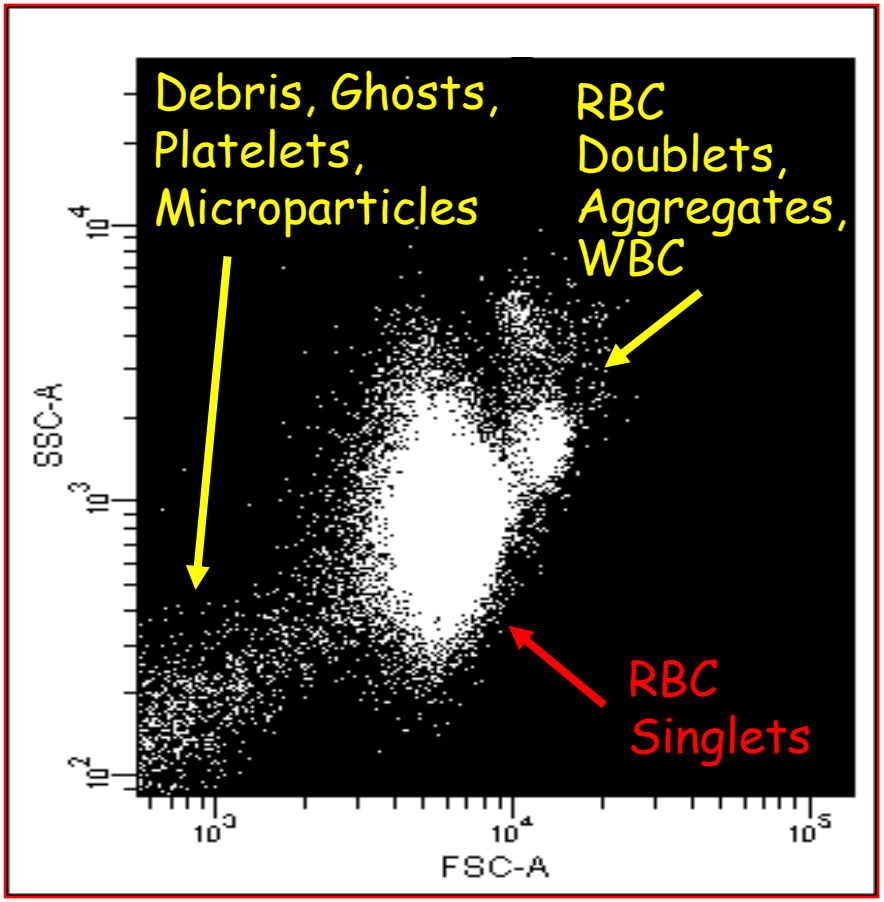


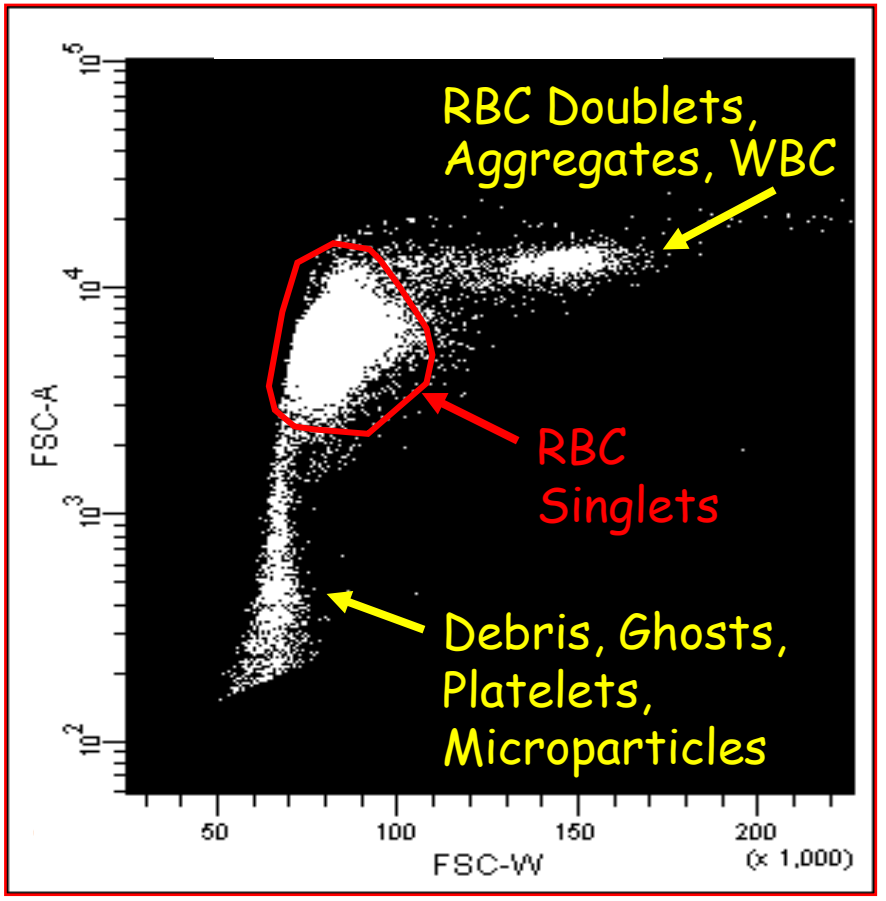






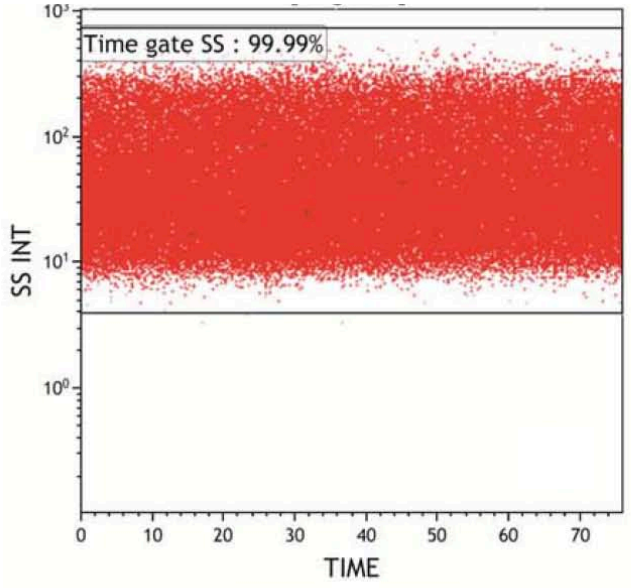
FCM Gating Strategy to Capture Peripheral Red Blood Cells

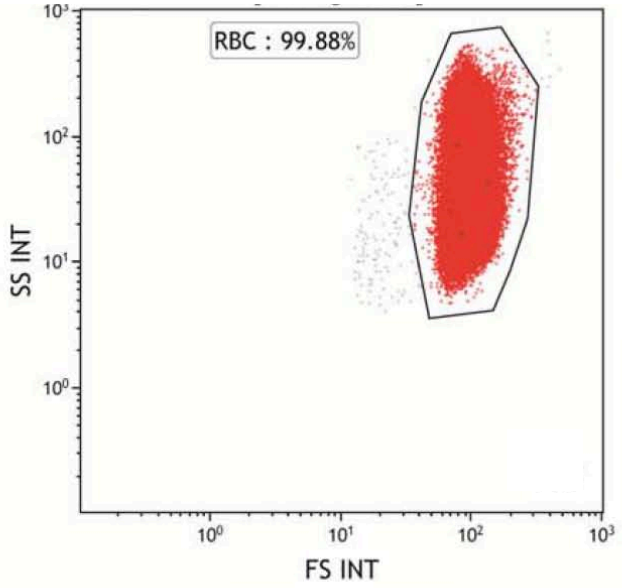


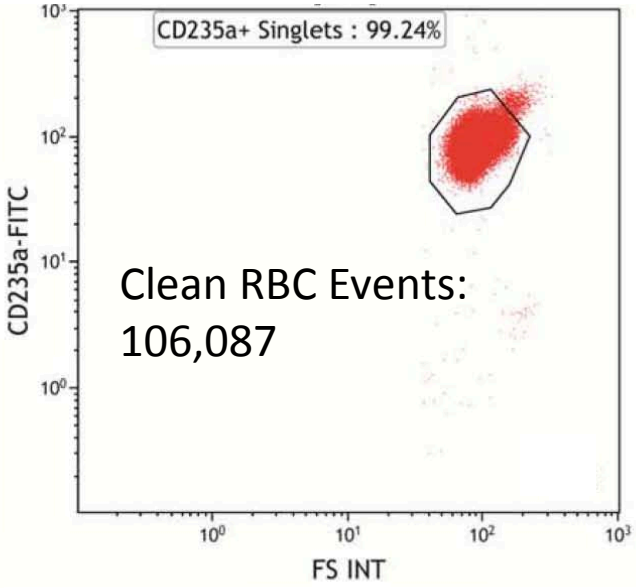


- Read at Low or Medium speed.
- Capture Gate: either using FSC Log /SSC Log (Left) or **better** with **Pulse-Analysis** (FSC-Width vs FSC-Area, Right).
- **Can avoid the usage of Glycophorin-A (CD235a) staining.**

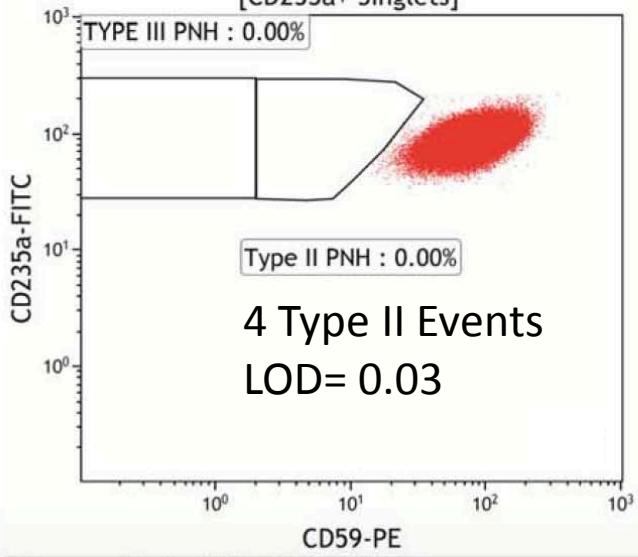
Validation of High-sensitivity PNH assay (Red Cells): Healthy Subject



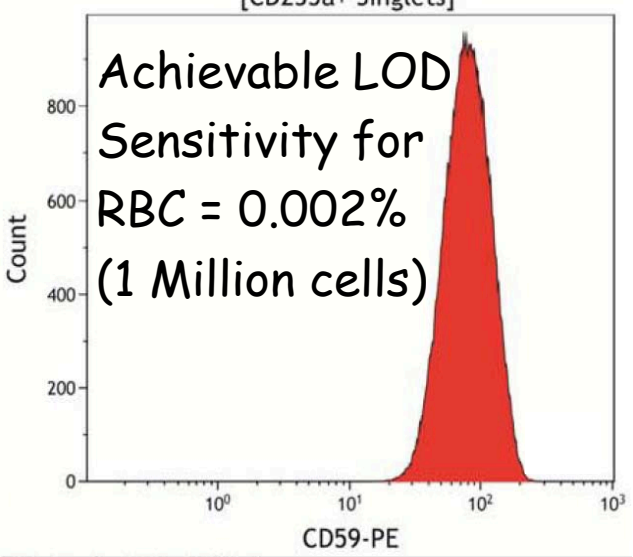




Clean RBC Events:
106,087



4 Type II Events
LOD= 0.03



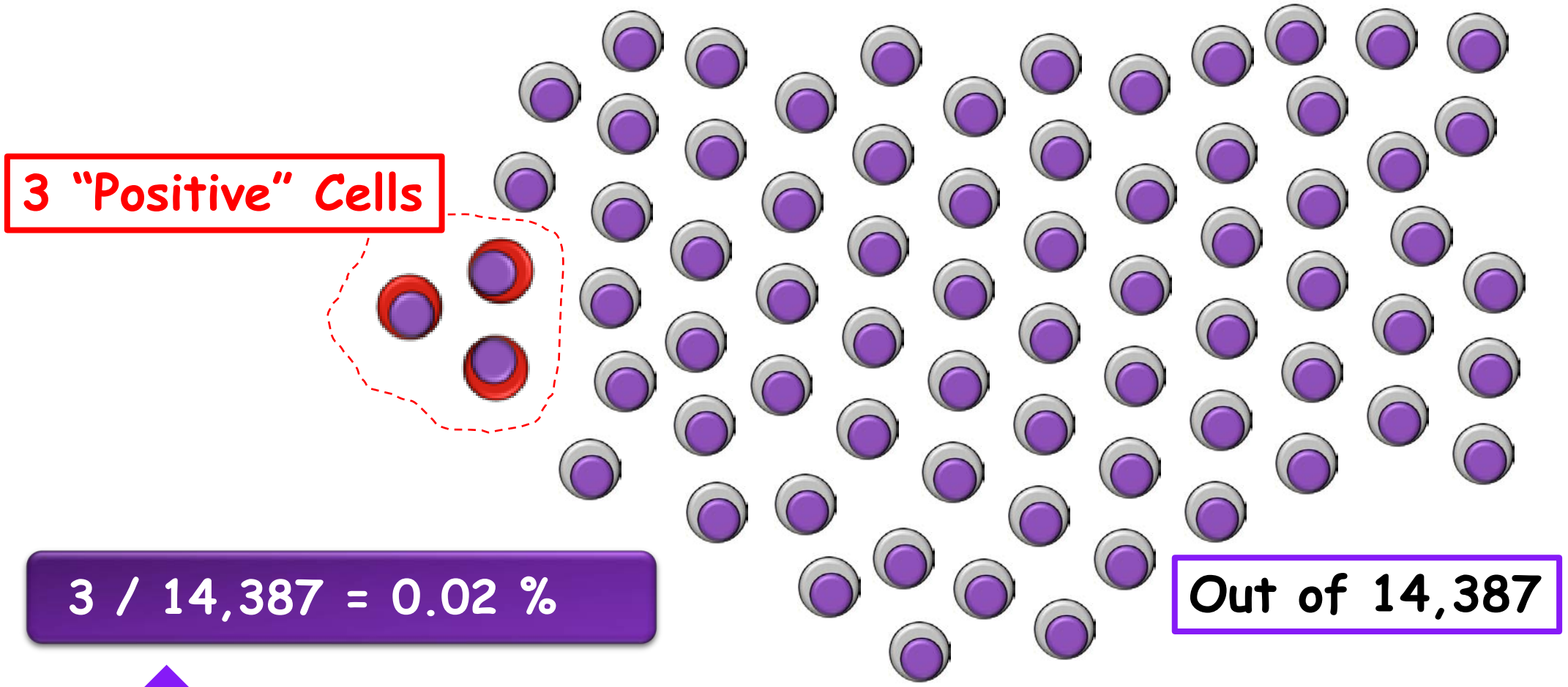
Achievable LOD Sensitivity for RBC = 0.002% (1 Million cells)

- Whole blood is diluted 1:100 with PBS.
- Incubation with CD59 plus CD235a (GLFa) for 20-30 min.
- Wash x2 with PBS + BSA.
- Read.

RBC are numerous: don't stop the run too early!

Illingworth A. Cytometry Part B (Clinical Cytometry) 2018; 94B: 49 – 66.

Rare Event Detection and Enumeration by FCM



3 / 14,387 = 0.02 %

Is it a correct approach? ➡ NO!

Enumeration of rare cell events (i.e. < 10^{-2}) **is NOT a mere arithmetical calculation.** Stringent technical and statistical criteria are required (both for 'positive' and 'denominator').

Rare Event and High-Resolution FCM Studies:

LOD & LOQ Vary According to the Total Number of Acquired Cells

Total Number of Acquired Cells (Excluding Erythroid)	LOD % ≥ 30 Events	LOQ % ≥ 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

Numerators

Denominators

The specific LOD 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

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).
The Sutherland/Illingworth group has adopted **20** events as LOD.

Illingworth A. Cytometry Part B (Clinical Cytometry) 2018; 94B: 49 – 66.

PNH High-Sensitivity Assay: Establishing the LLOQ

According to the Total Number of Acquired Cells

Total Number of acquired cells	LLOQ % ≥ 50 Events
5.000	1%
50.000	0,1%
500.000	0,01%

Think about Monocytes

- It is very difficult to acquire more than 20,000-30,000 Monocytes in most instances (**LOQ 0.1%** the highest attainable sensitivity).
- Under normal analytical conditions it is almost **impossible** to acquire 500,000 Monocytes (**LOQ 0.01%**)!!
- **20 Type III Neutrophil Events** and CD15 can be trusted (the same with Type II Monocytes and without CD64 is highly unreliable!).

UK NEQAS PNH Survey 2017

Variation in Gated Events for Neutrophils, Monocytes and RBC

How many gated events are counted?	Granulocyte (n = 88)	Monocyte (n = 69)	RBC (n = 78)
4	0	1	0
100	2	1	1
1,000	0	3	0
2,000	0	1	0
3,000	0	1	0
5,000	5	7	1
10,000	9	11	5
15,000	1	0	1
20,000	4	1	4
25,000	1	0	0
30,000	8	8	1
50,000	13	5	15
100,000	22	15	19
150,000	2	2	1
200,000	3	2	2
250,000	7	4	6
300,000	6	3	8
500,000	4	3	12
1,000,000	1	0	2
2,500,000	0	1	0

Reported Level of Sensitivity (*LOQ ?*)

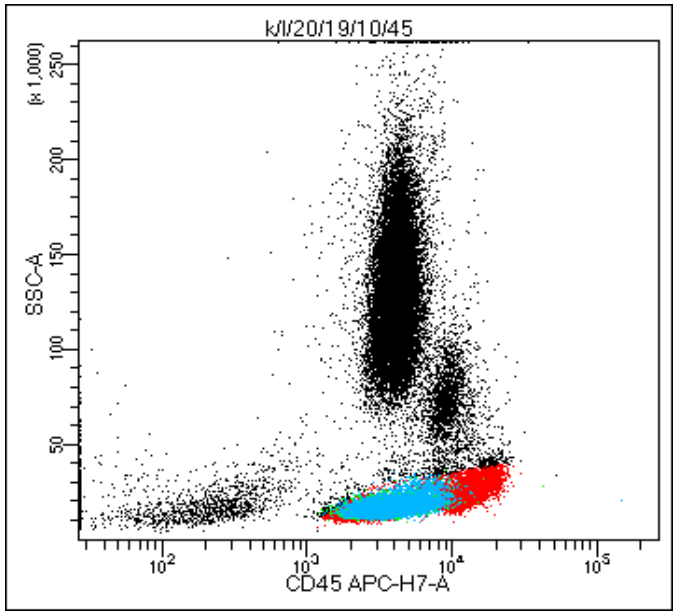
Population size at which considered clone present	Granulocyte (n = 87)	Monocytes (n = 70)	RBCs (n = 75)
<0.01%	4	3	3
0.011–0.05%	26	21	23
0.051–0.1%	12	6	10
0.11–0.5%	13	12	13
0.51–1%	15	12	15
1.1–5%	17	12	11
>5.0%	0	4	0

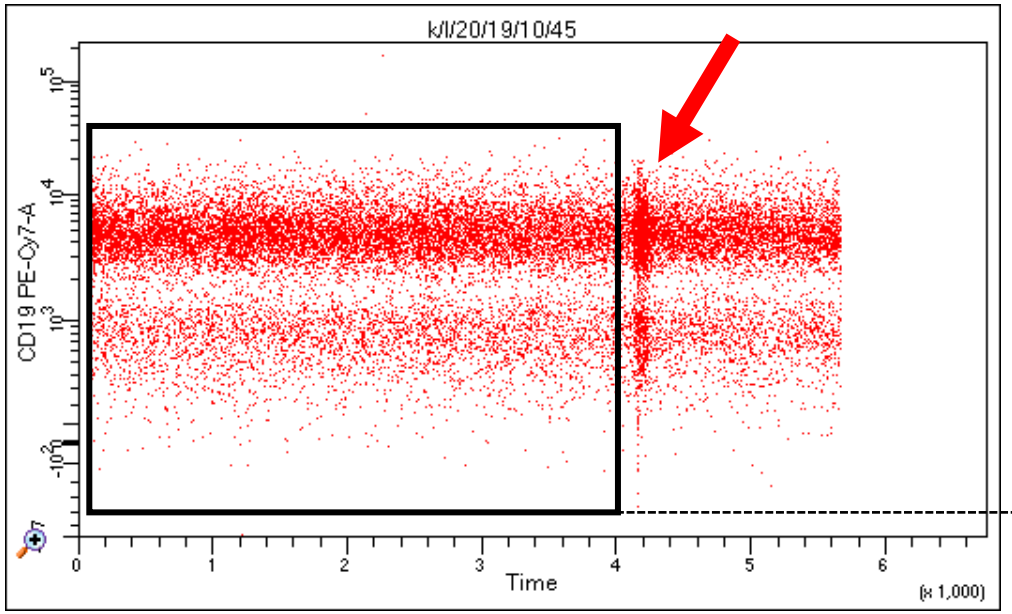
Some centers **BELIEVE** they are working at a certain level of sensitivity.

0.011 % for Monocytes implies the acquisition of 500,000 cells !

Fletcher M. Cytometry Part B. 2017; 92B: 266-274.

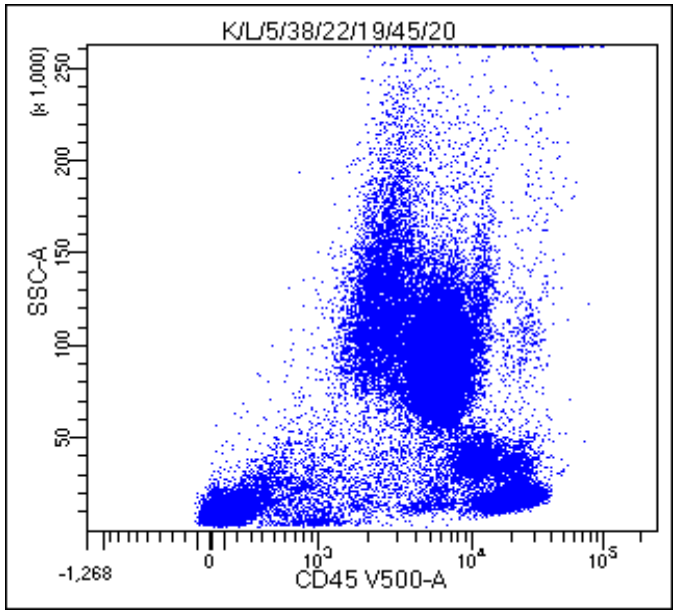
If you don't look at the TIME Parameter, you may not be aware of cell flow troubles

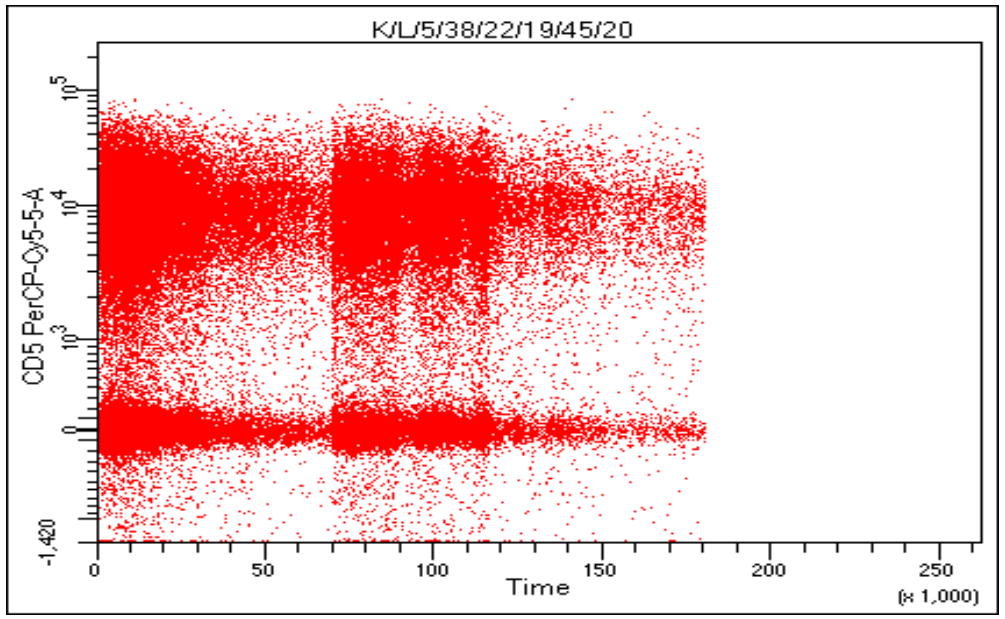




Apparently a good run, but a perturbation has been registered.

Restrict the acceptance of the events with a gate on TIME.

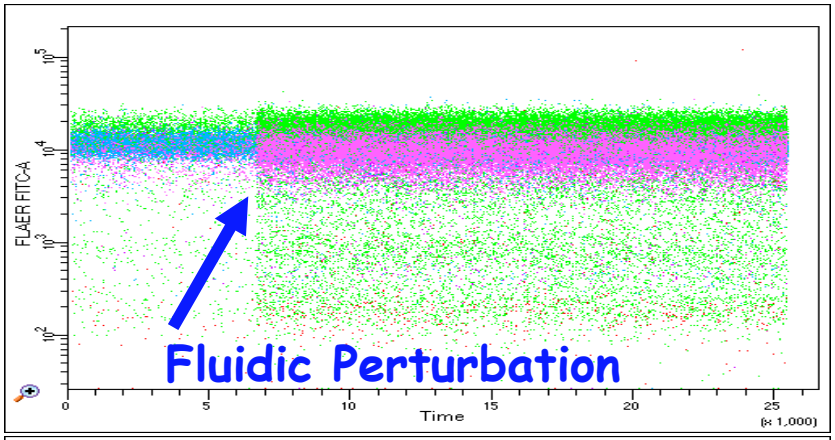


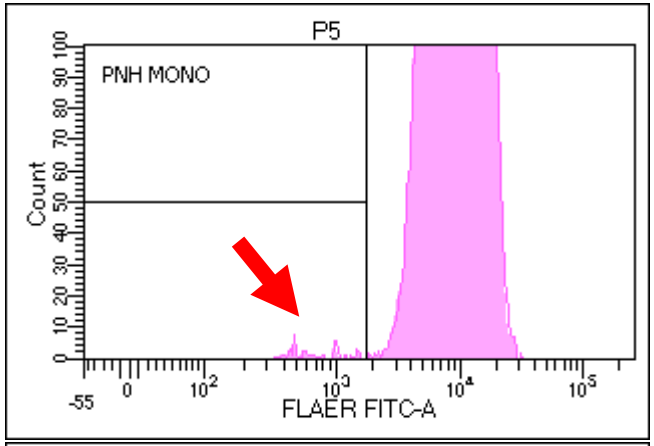


Apparently not a bad run (just a little blurred)

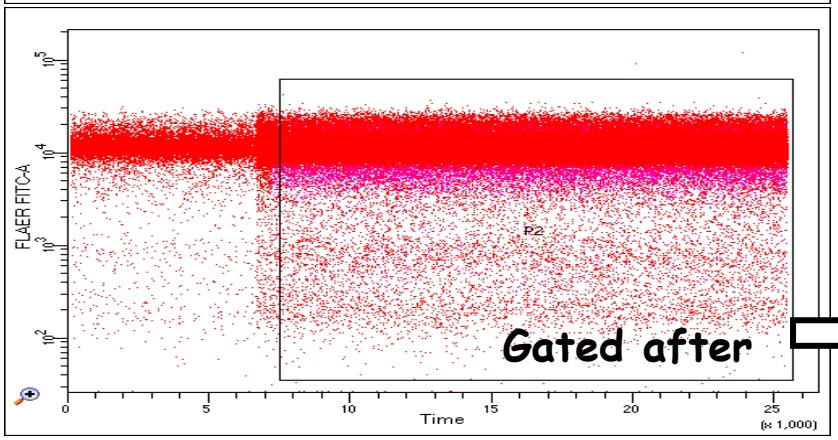
In fact, a badly perturbed run, to be rejected.

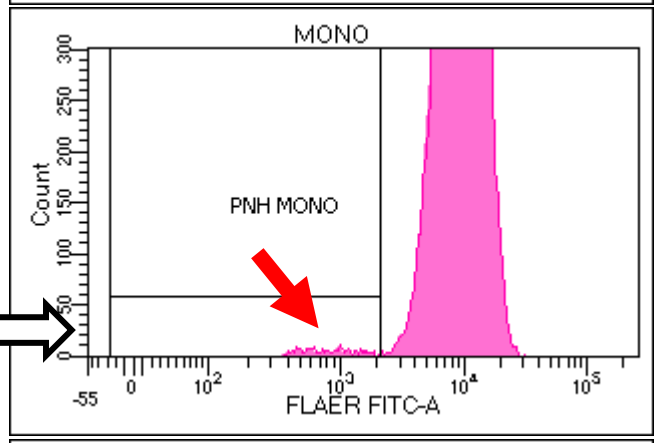
Small PNH Clones: Artifacts Produced by Fluidic Perturbations



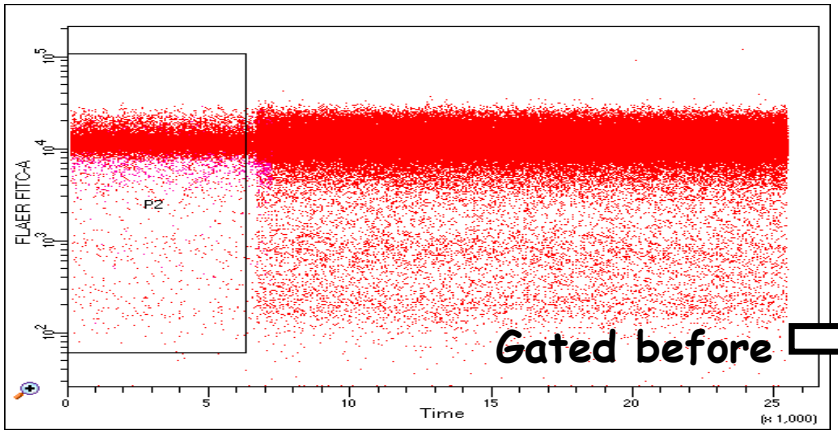


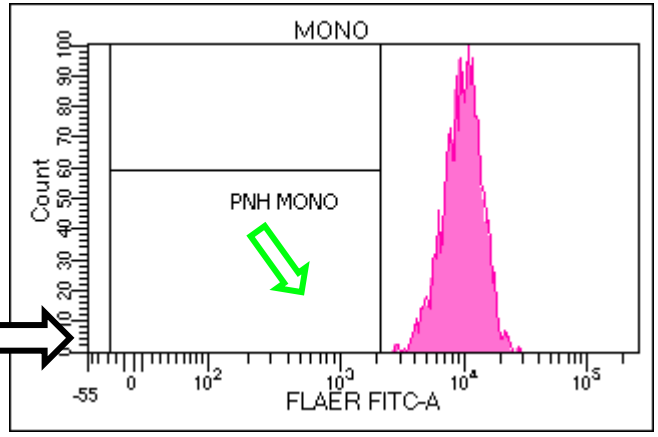
A small Monocyte PNH clone seems present. The TIME display shows however the occurrence of a fluidic perturbation





The putative PNH events are generated AFTER the occurrence of the perturbation.

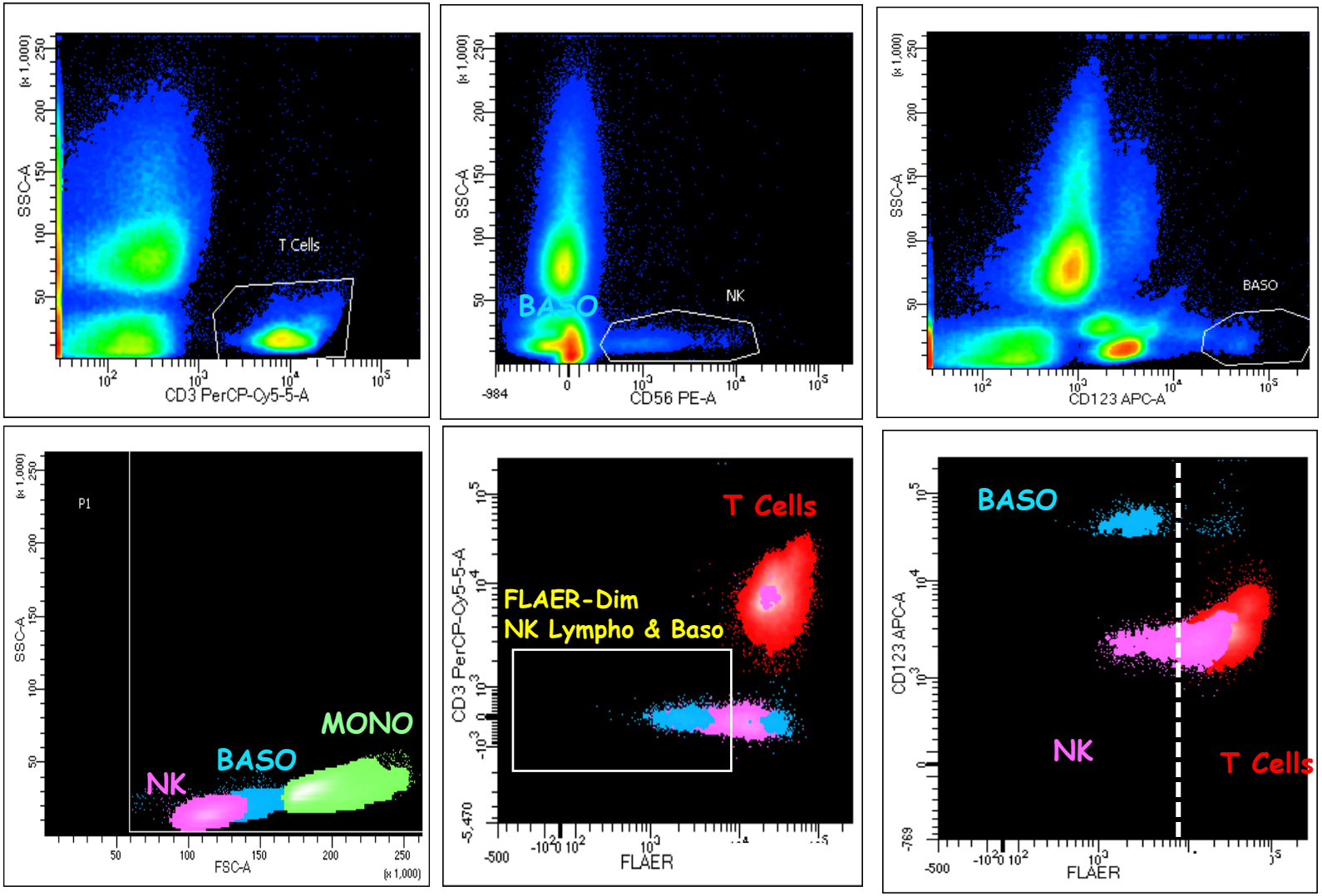




Restricting the gate to the events BEFORE the perturbation, the false PNH events disappear.

Normal Donor – Managing The FLAER-Dim 'Lymphocyte' Events

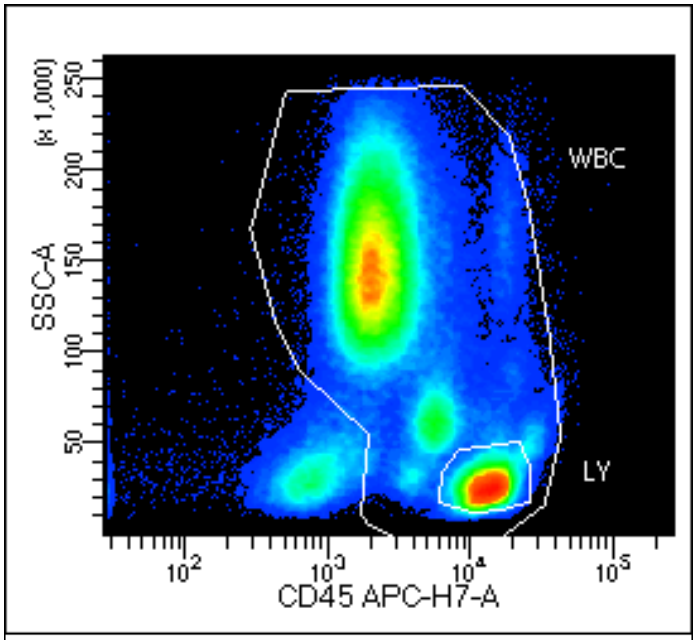
Artifacts are always possible !

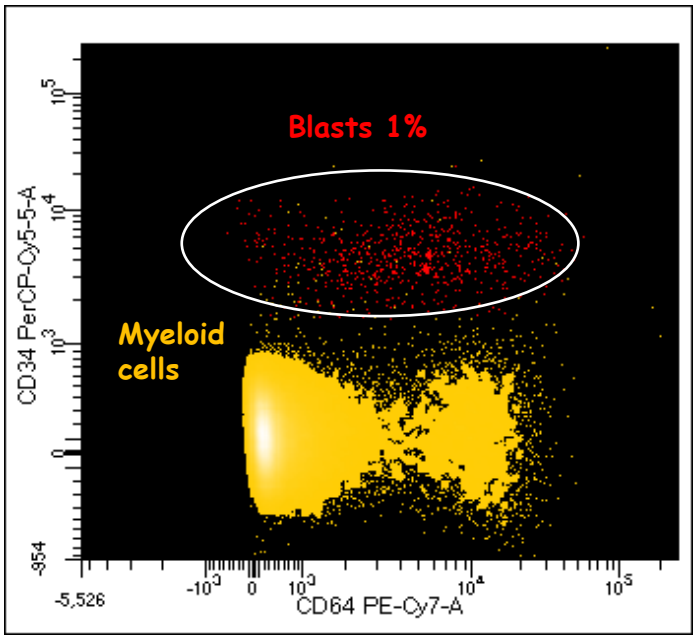


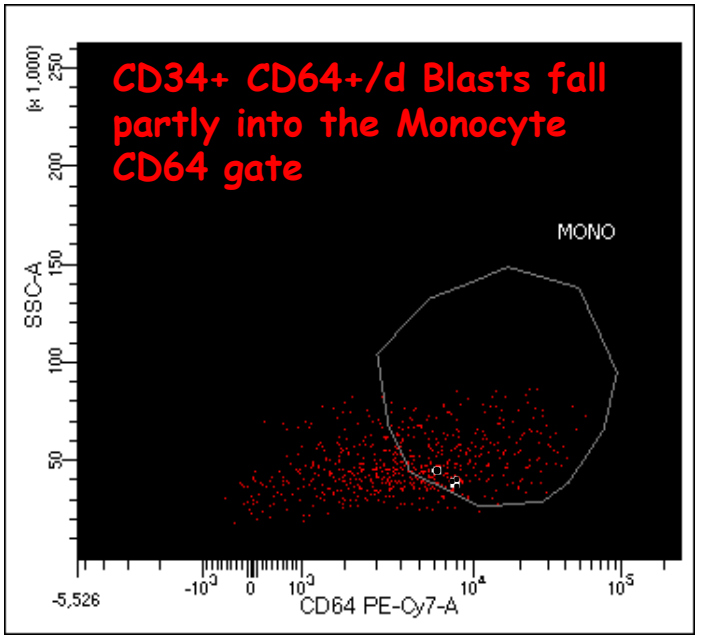
FLAER-Dim events in normal subjects can be generated by NK Lymphocytes and Basophils

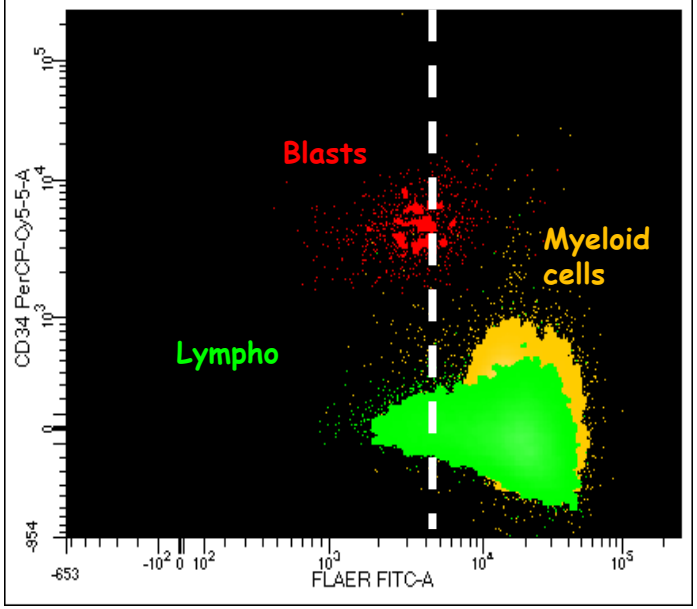
PATIENTS: Small FLAER-Dim Clusters that may cause troubles (Blasts)

Artifacts are always possible !





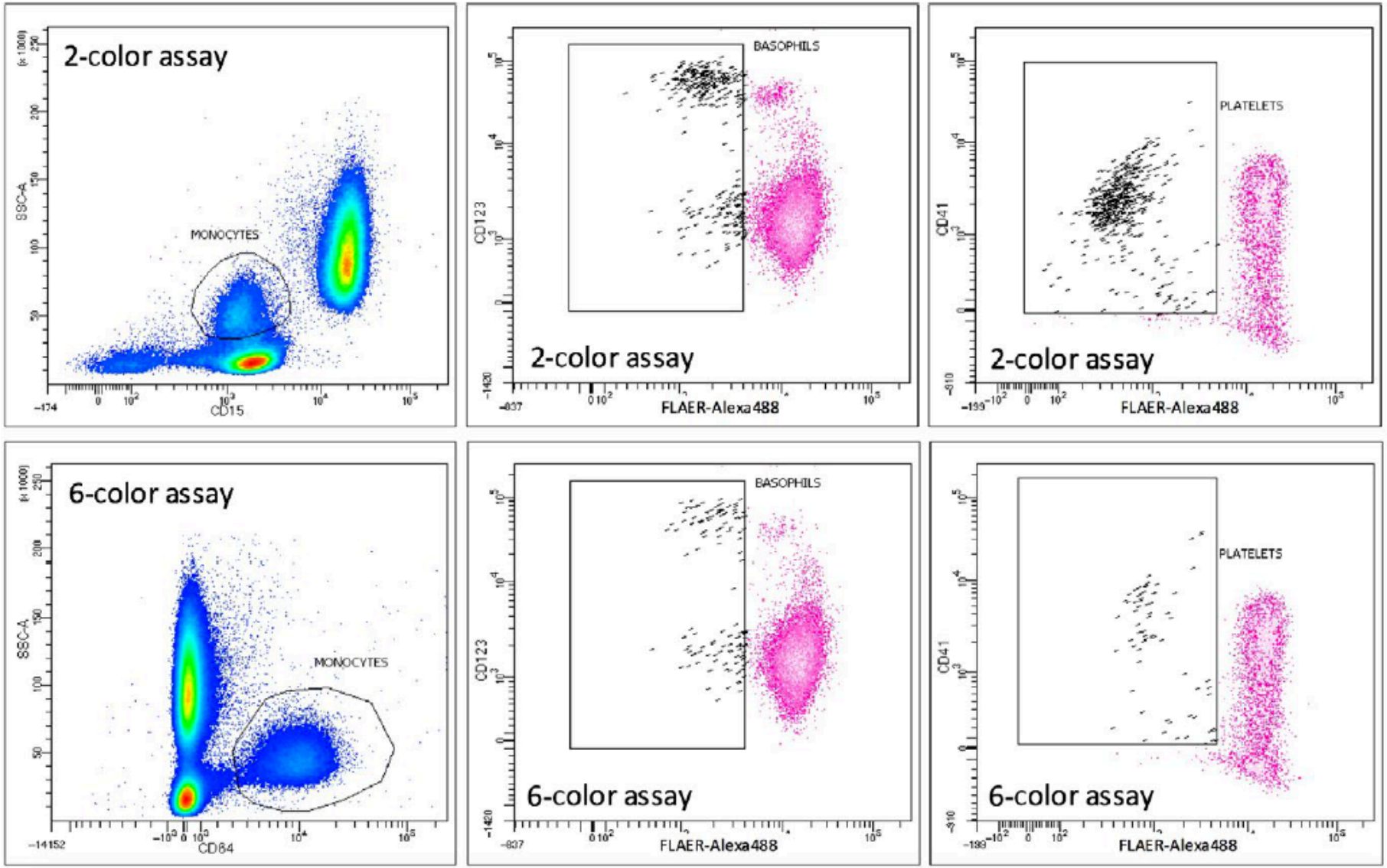




CD34+ CD64+ Cells (From an AML in relapse) may simulate the presence of a small FLAER-Dim cell population.

Platelets, Basophils, Dendritic Cells as Sources of Artifacts Also in Hi-Res PNH Analysis

(Contamination of Monocyte Gate)



The Same Sample
The Same Datafile

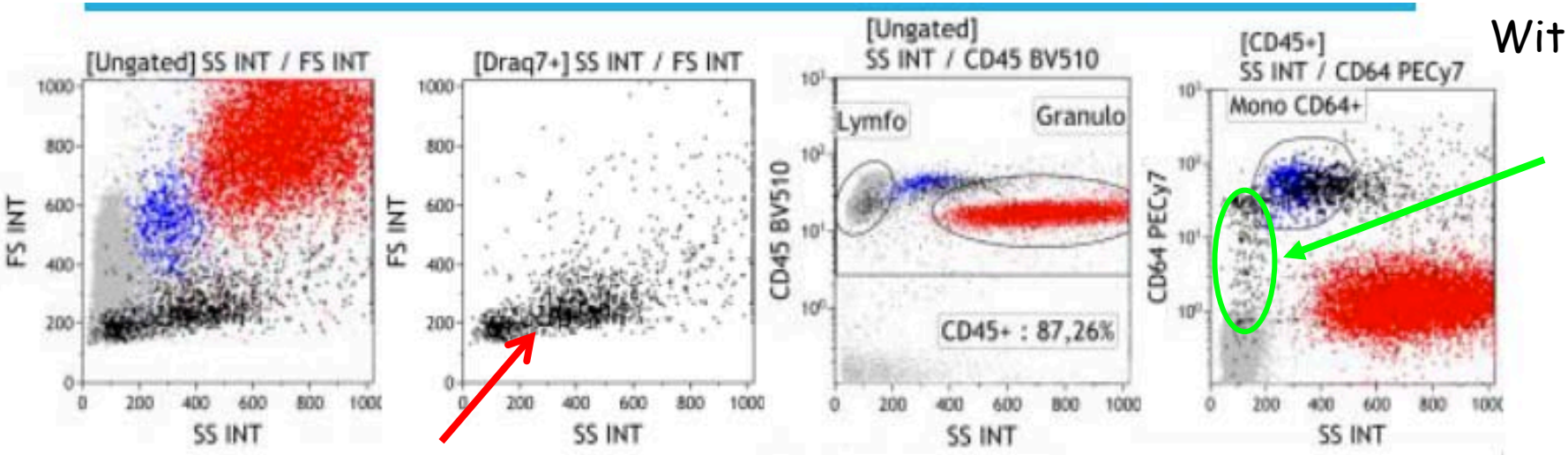
2 Color Gating

6 Color Gating

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

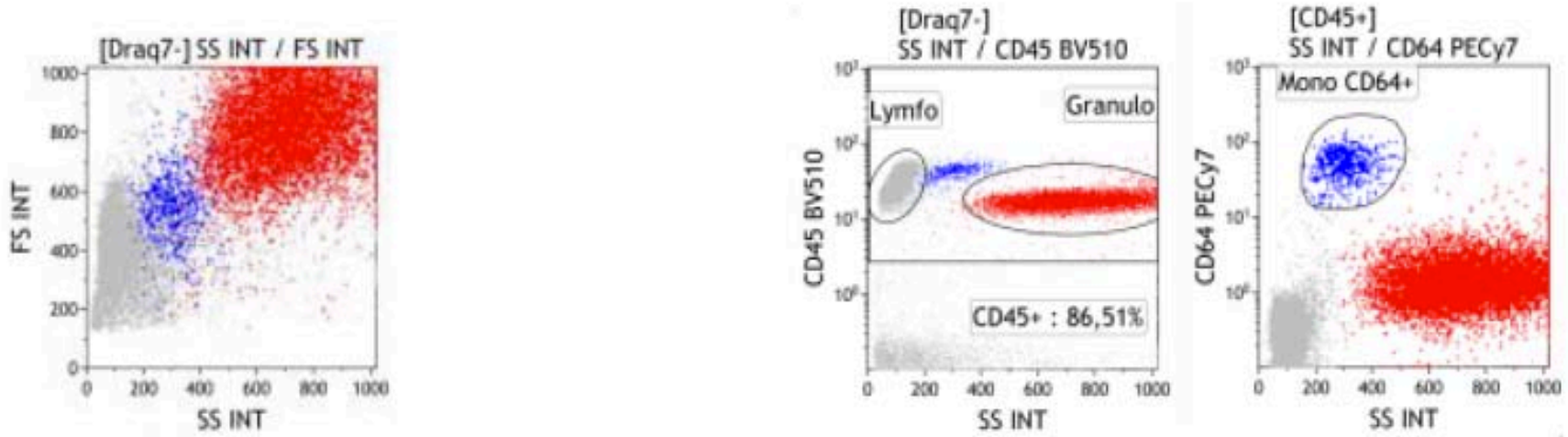
Influence of Dead or Apoptotic Cells

Without DRAQ7



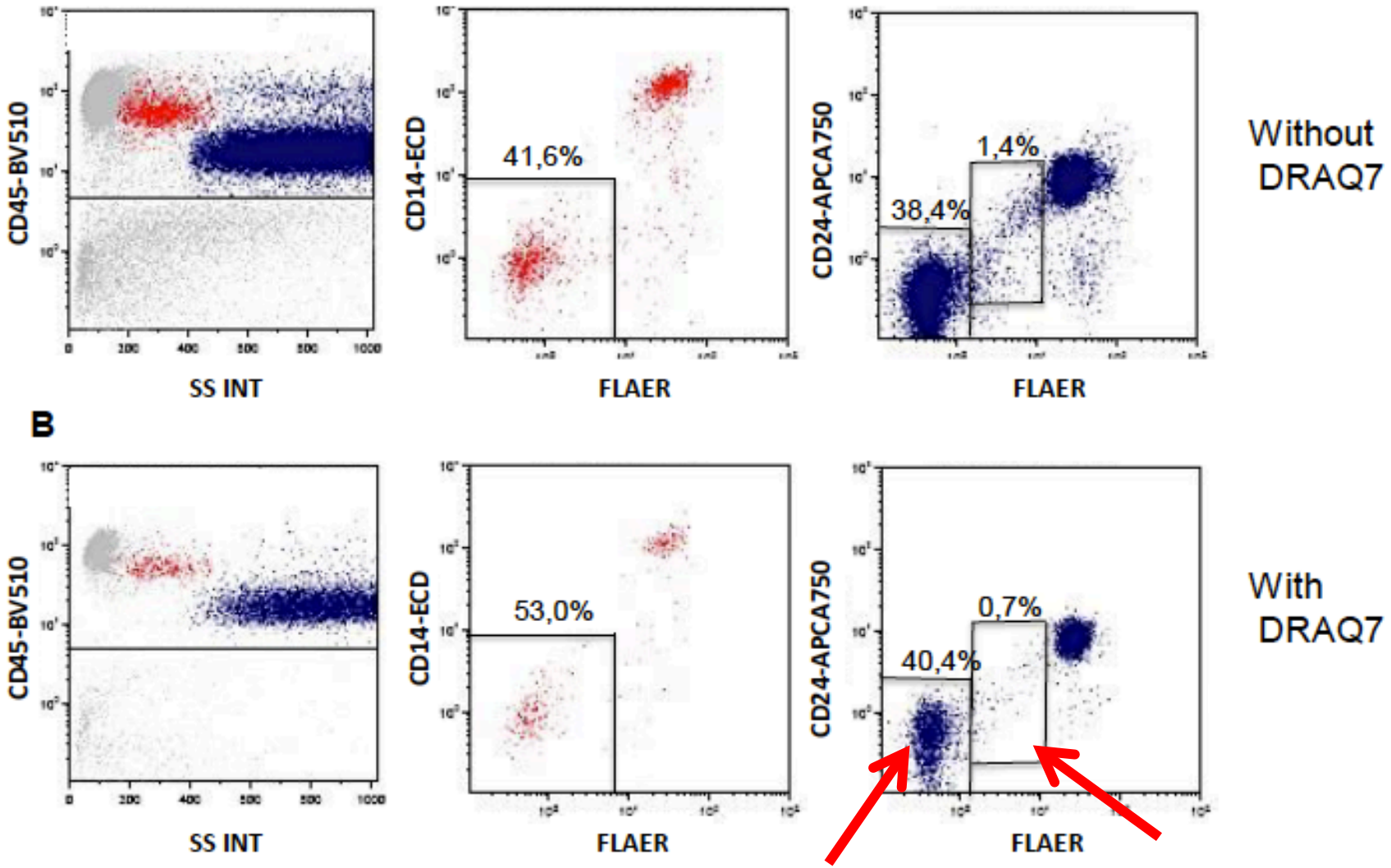
Dead and apoptotic cells can non-specifically stain dimly with MoAbs and FLAER

With DRAQ7



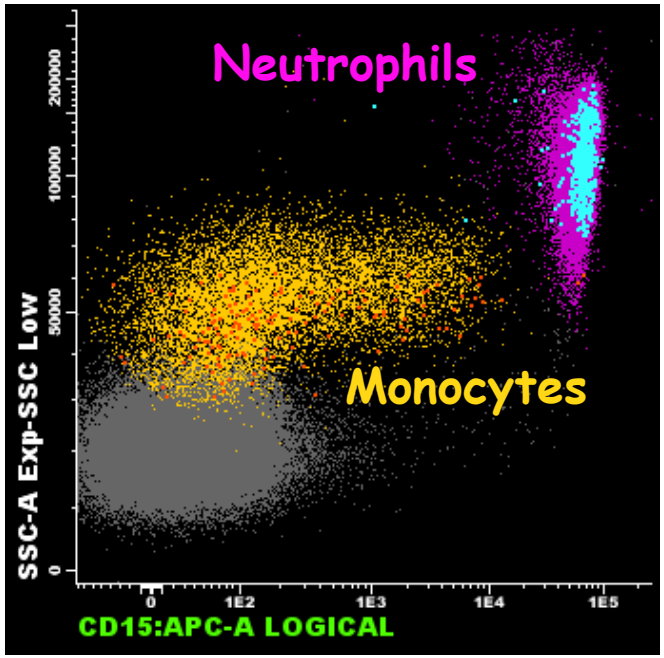
Courtesy of F. Preijers 2016

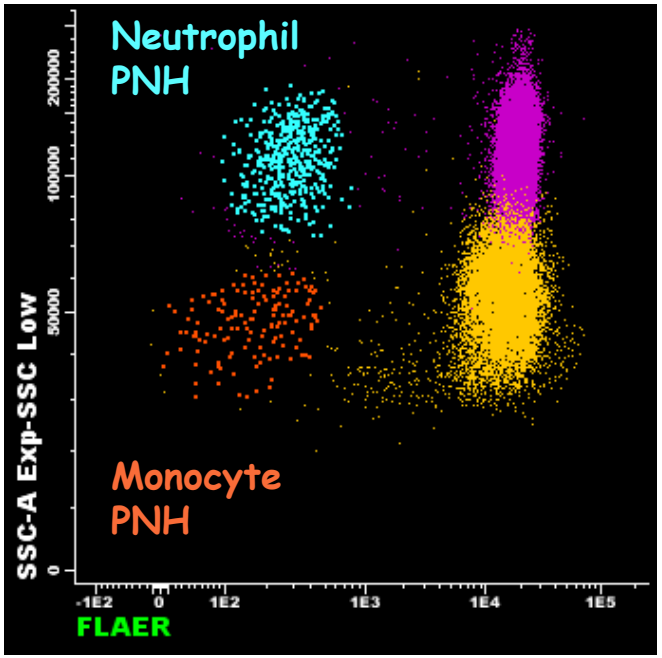
Influence of Dead and Apoptotic Cells on PNH Analysis

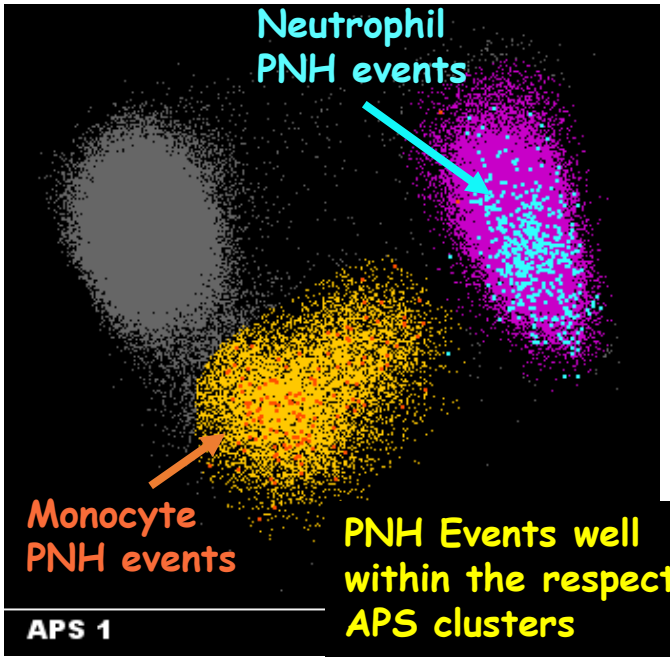


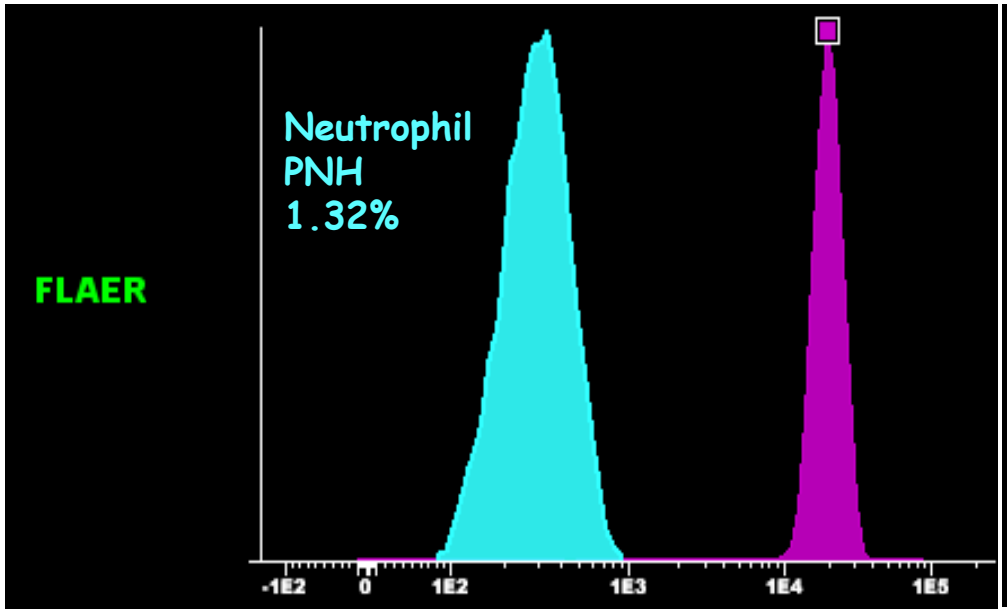
Courtesy of F. Preijers 2016

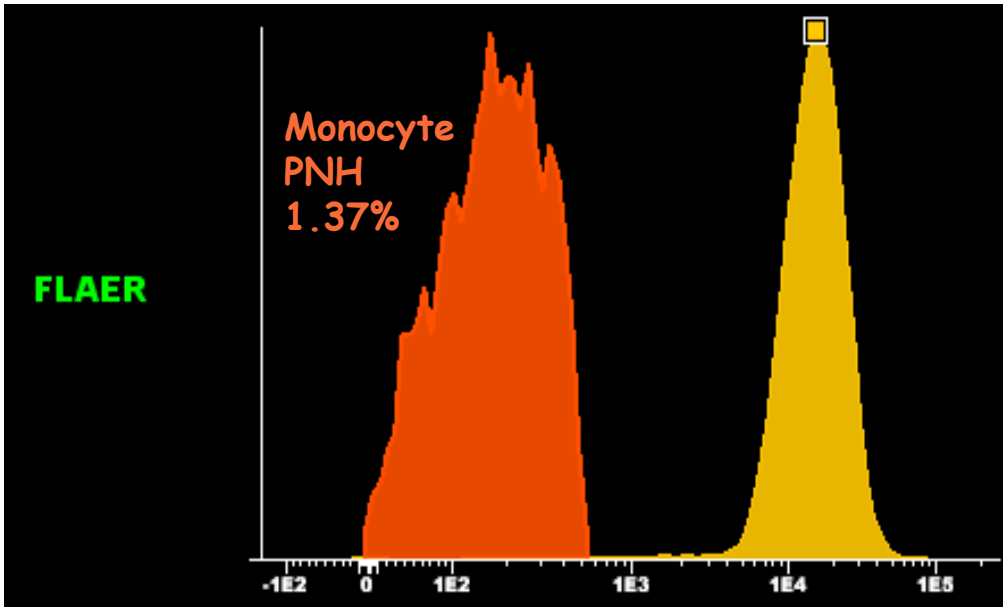
PNH Analysis Using Infinicyt™ and APS (Principal Component) Clustering



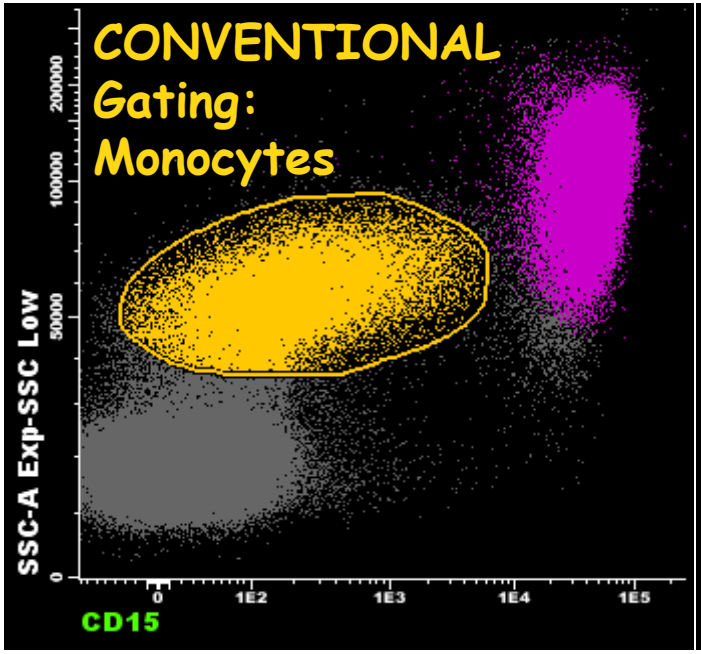


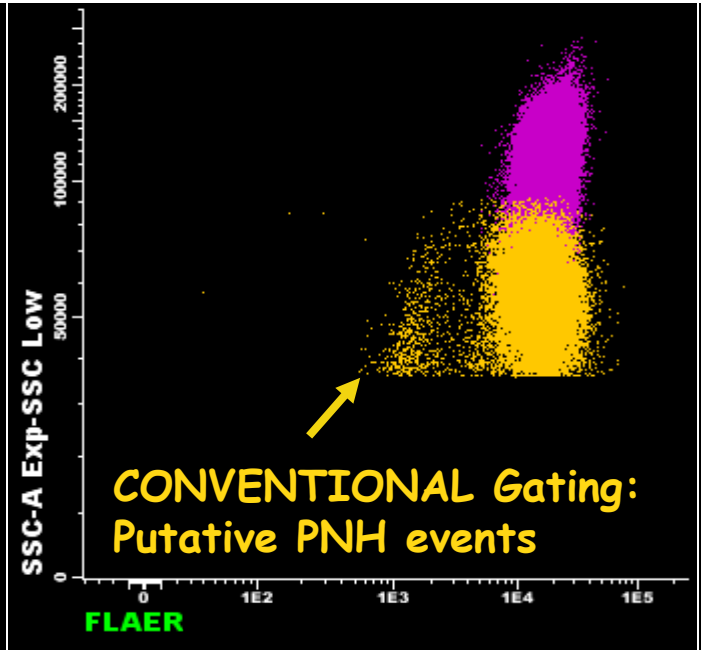


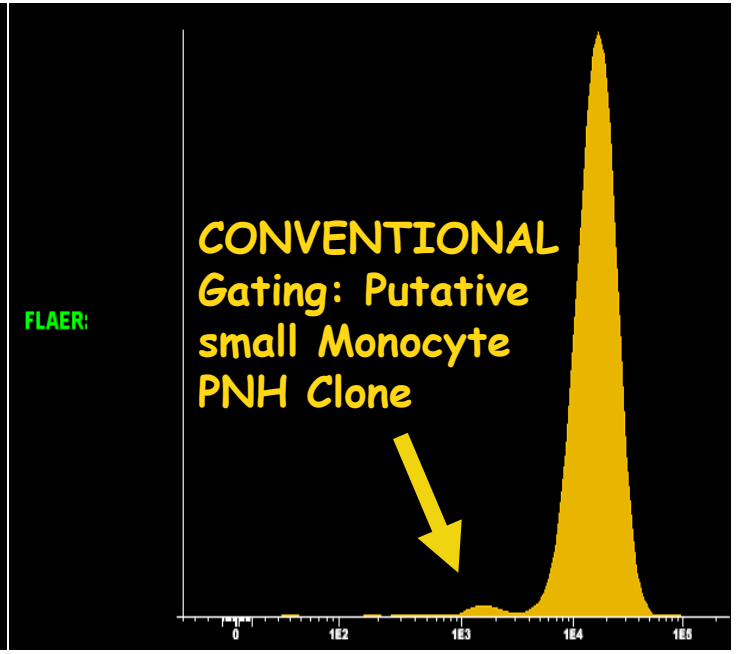


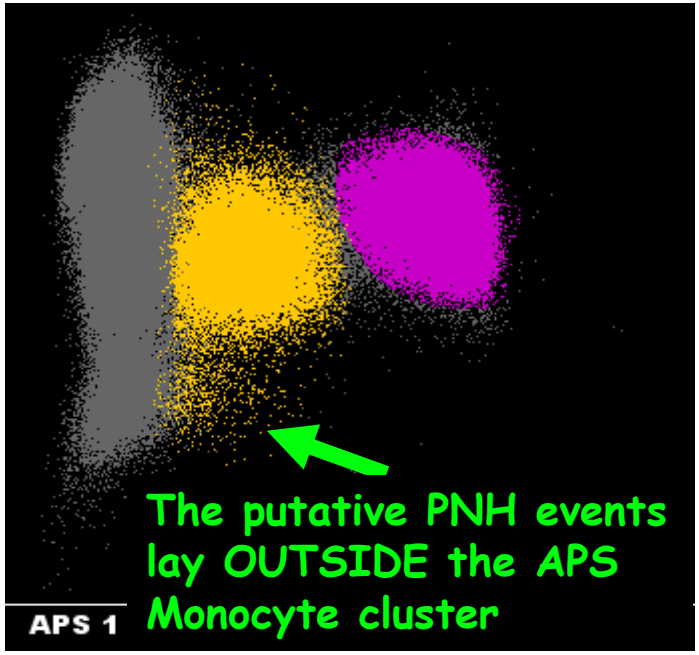


PNH Analysis Using Infinicyt™ and APS (Principal Component) Clustering









The **conventional** gating provides only a **bi-dimensional** selection of cell clusters.
Undesired events can be inadvertently dragged in.

The **APS – Principal Component** clustering reflects the **multi-dimensional** positioning of a given cell cluster.
Undesired events such as PNH artifacts can be recognized more easily.

UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Paroxysmal Nocturnal Haemoglobinuria

Sample - 097

Red Blood Cell PNH Clone

Exercise 097: PNH Clone ABSENT

Flow Cytometer	Returns	Laboratories Reporting Clone Present	Laboratories Reporting Clone Absent	Median Clone Size (%)	Lower Quartile (%)	Upper Quartile (%)
Facs Canto II	68	3	65	0.00	0.00	0.00
Navios	28	3	25	0.00	0.00	0.00

False positive RBC PNH Clones (6/96)

Monocytes PNH Clone

Flow Cytometer	Returns	Laboratories Reporting Clone Present	Laboratories Reporting Clone Absent	Median Clone Size (%)	Lower Quartile (%)	Upper Quartile (%)
Facs Canto II	59	1	58	0.00	0.00	0.00
Navios	25	1	24	0.00	0.00	0.00

False positive WBC PNH Clones (2/84 – 4/104)

Granulocytes PNH Clone

Flow Cytometer	Returns	Laboratories Reporting Clone Present	Laboratories Reporting Clone Absent	Median Clone Size (%)	Lower Quartile (%)	Upper Quartile (%)
Facs Canto II	75	3	72	0.00	0.00	0.00
Navios	29	1	28	0.00	0.00	0.00

UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Paroxysmal Nocturnal Haemoglobinuria

Gating Antibodies

Results Input Webpage – Example: GRANULOCYTES

Antibody Used	Antibody Manufacturer	Fluorochrome
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **

GPI Linked Antibodies

Antibody Used	Antibody Manufacturer	Fluorochrome
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **
** Please Select **	** Please Select **	** Please Select **

Please state your level of sensitivity for this cell population:

This box must be filled with the ACTUAL sensitivity level achieved for this specific sample

REPORTING

NO PHENOTYPIC EVIDENCE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

Comment: Flow cytometric analysis does not show any evidence of a PNH clone based upon analysis of a variety of GPI-linked antibodies on red blood cells, monocytes and neutrophils. These findings do not support a diagnosis of PNH. Clinical correlation is recommended.

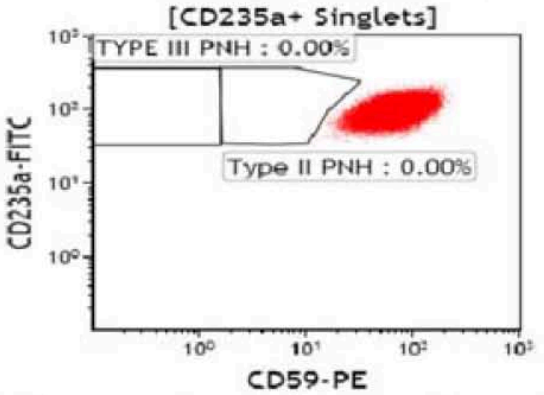
Reference: 1. Borowitz et al: Guidelines for the Diagnosis and Monitoring of PNH and Related Disorders, Clin Cytometry 2010, 211-230
2. Sutherland et al: Practical guidelines for the high-sensitivity detection and monitoring of PNH clones by flow cytometry. Cytometry B Clin Cytom 2012; 82:195-208.
3. http://www.pnhsource.com/physicians

Illingworth A. Cytometry Part B 2018; 94B: 49 – 66.

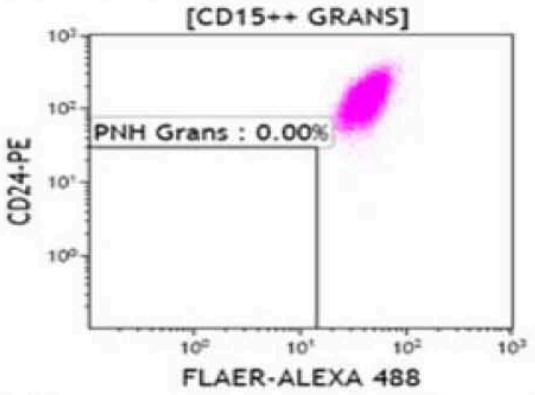
Flow Results: ***Immunophenotypic analysis*** was performed using gating antibodies CD45, CD15, CD64, CD235a, GPI-linked antibodies CD59, CD14, CD24 and FLAER.

Cell population	Result	LLOQ
CD235a+ RBC	No CD59 negative RBCs identified	0.01%
CD64+ Monocytes	No FLAER/CD14-negative cells **50,000 acquired?**	0.1% *
CD15+ Neutrophils	No FLAER/CD24-negative cells	0.01% *

The Lower Limit of Quantitation (LLOQ)* of WBC assay may be decreased in severely pan-cytopenic patients.



Sample histogram of a typical patient with no evidence of PNH in RBC's



Sample histogram of a typical patient with no evidence of PNH in Neutrophils

The ACTUAL number of clean acquired events for each population should be included, to complete the report

REPORTING

Recommended Terminology for PNH **(CLSI document H52-A2)**

PNH Population	Type
Greater than 1%	PNH clone
0.1% - 1%	Minor PNH clone or population of PNH Cells
< 0.1% and below	Rare cells with PNH phenotype

It is important to not over-interpret small PNH clones as Clinical PNH.

Detecting a SMALL PNH clone does NOT mean that a patient has a clinical PNH.

Davis BH. CLSI H52-A2 Red Blood Cell Diagnostic Testing Using Flow Cytometry. Approved Guideline, 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute. 2014. ISBN Number: 1-56238-957-2.

Annals of Hematology

Features, Reason for Testing and Changes with Time of 583 Paroxysmal Nocturnal Hemoglobinuria Clones from 529 Patients: a Multicenter Italian Study

--Manuscript Draft--

Manuscript Number:	AOHE-D-17-00835R2	**R2 Submitted 16 November 2018**

By: Elisa Cannizzo (and 45 Co-Authors)

- Type II PNH clones can be found in about a half of cases with >50% Type III Clones.
- Type II PNH clones are very rare when Type III clones are smaller (i.e. <10%).
- Small PNH clones are mostly associated with BM Failure and MDS.
- **Pediatric** PNH cases have mostly **small** clones.
- About **6.3%** of small PNH clones can INCREASE over 1-year follow-up.
- About **15%** of small-to-medium PNH clones can DECREASE over 1-year follow-up.

Annals of Hematology

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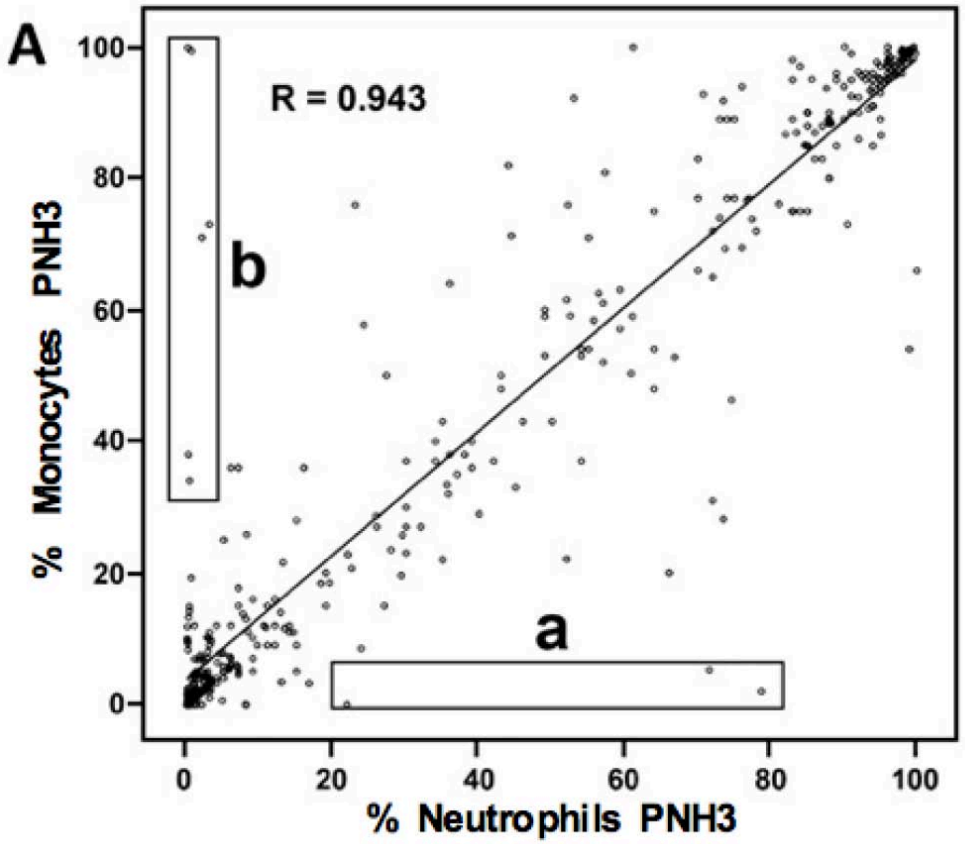

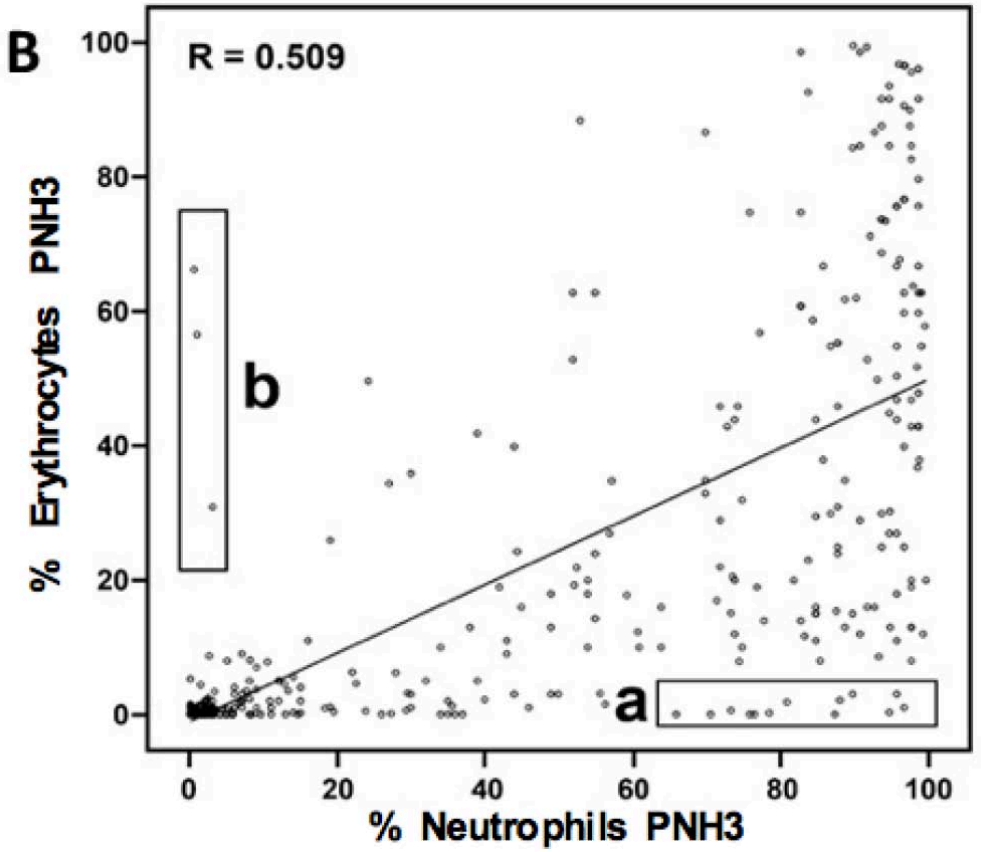

Managing Sub-Clinical PNH Clones by High-Sensitivity FCM Analysis

Conclusions

- The same cytometric criteria and rules used in rare event and Minimal Residual Disease analysis should be applied in the study of small PNH clones.
- Each lab should establish strict criteria to define TRULY NEGATIVE cases and the BACKGROUND EVENTS for RBC, Neutrophils and Monocytes.
- Bulk Lysis technique is of great help to increase the Signal-to-Noise ratio.
- The sensitivity level to be reached is not an independent value, but rather VARIES from sample to sample according to the total clean events captured.
- Usage of TIME parameter is mandatory to monitor the regularity of the run. Fluidic perturbations are a major source of artifacts (i.e. 'small PNH events').
- Do not overemphasize 'small Type II PNH clusters', especially with Monocytes and without an accompanying larger Type III clone.
- Principal Component Analysis can be of help in establishing the 'TRUENESS' of small PNH clones.