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Identification of pre-analytical errors

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Conflict of Interest Disclosure

In accordance with criterion 24 of document UEMS 2012/30 "Accreditation of Live Educational Events by the EACCME®" we herewith declare to have submitted a Conflict of Interest Disclosure Form to ESCCA.

This COI Disclosure Form can be viewed at the ESCCA 2019 Conference website www.escca.eu/norway2019 - Programme section / Accreditation page

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Flow Analysis Today

Flow cytometric immunophenotyping anno 2019

- Sophisticated multi-color FCMs

- Bright fluorochromes

- Highly specific MoAbs

More colors requires more plots, events, protocol complexity and ultimately increased analysis time

What are the pitfalls???

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Essentials for Immunophenotyping of leukemia and lymphoma

How do you differentiate between normal and malignant populations

- **Percentage / Number of aberrant cells**
- **Pattern recognition:**
  - What is the normal pattern of expression?
  - What is the aberrant pattern of expression?

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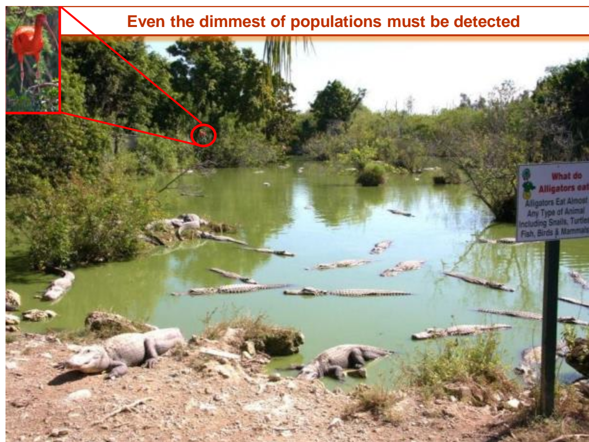
Even the dimmest of populations must be detected



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??Tip and tricks for discrimination between cells
By using multi-colour flow cytometry??

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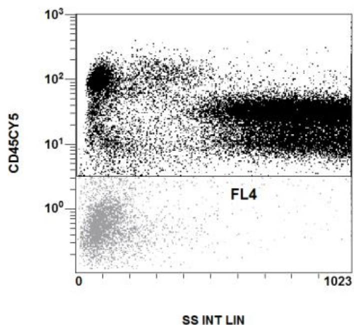
CD45/SS plot BM: which populations do I see and is the content correct?



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What can happen in cell discrimination??

A few disasters:

- Auto fluorescence (UV > red laser, myeloid/ erythroid…)
- Fc receptor binding (mono, granulo, B cell, T cell)
- Dead/apoptotic cells
- Non-specific binding of conjugates to cells
- Debris (RBC, microparticles, cell fragments, ..)
- Spectral overlap, dissociation of label (PECy7)
- Sample carryover
- Etc. etc.

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What do we need for a robust multi-colour immunophenotyping?

- ***What should be the composition of the panel/combination of MoAb-conjugates?***
- ***How many colors should be used? More is not always better, but is more complicated***

Starts your IPT with *"Which question should be answered" or "which populations should be identified by which antigens"*

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What do we need for multi-colour immunophenotyping?

- ***Specific MoAbs (CD1-CD371 [updated HLDA10, Sydney]***
- Bright Fluorochromes
- Excellent (tandem) conjugates
- Pre-analytical prerequisites
- Highly sensitive Flow Cytometers
- Technical procedures

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Monoclonal antibodies (CD's)

"Leucocyte Typing Workshop" (HLDA)

	Workshops	Number of MoAb	CD-codes determined
I	Paris, 1982	±150	CD1-CDw15
II	Boston, 1984	±350	CD16-CDw26
III	Oxford, 1986	±900	CD27-CD45
IV	Vienna, 1989	±1100	CD46-CDw78
V	Boston, 1993	±1450	CD79-CDw130
VI	Kobe, 1996	±1150	CD131-CD166
VII	Harogate, 2000	±270	CD167a-CD247
VIII	Adelaide, 2004	±100	CD248-CD339
IX	Barcelona, 2010	???	CD339-CD363
X	Sidney, 2014	???	CD364-CD371
XI	Pending	???	

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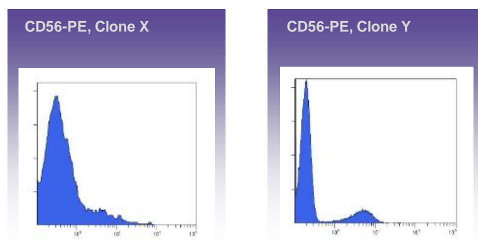
Specific MoAbs: clone selection



Clone Selection Based on Performance.

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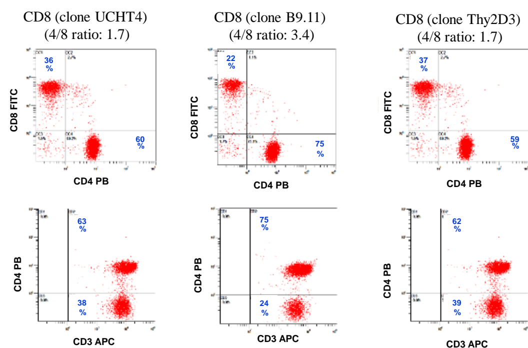
Which CD8 clone is the correct one to use?



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Which clone is the correct one to use?

Effect in CD8 also found in other CD's but also in different conjugates of one clone (e.g. CD19)

How to solve this problem?

1. Test more MoAbs clones
2. Use always the same clone/conjugate in your IPT
3. In multicenter studies: Use the same conjugates
4. Titrate all MoAbs and determine optimal S/N and not maximum MFI

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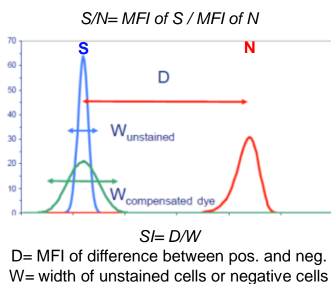
Signal-to-Noise ratio versus *Staining index*

S/N= MFI of S / MFI of N



SI= D/W

D= MFI of difference between pos. and neg.
W= width of unstained cells or negative cells

- Negative population should be stained with same MoAb conjugate
- Negative population must be representative for the positive population
- E.g. CD8+ and CD8- T cells (NOT: T cells and neutrophils)

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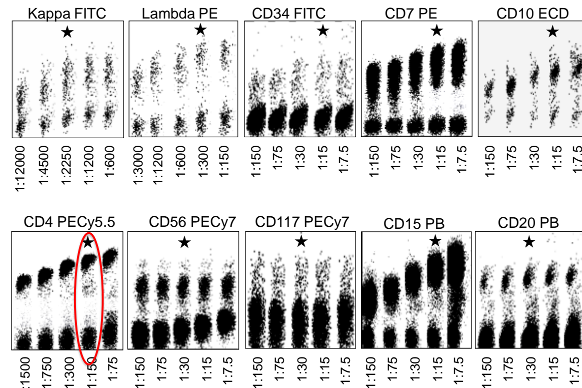
MoAbs must be titration!



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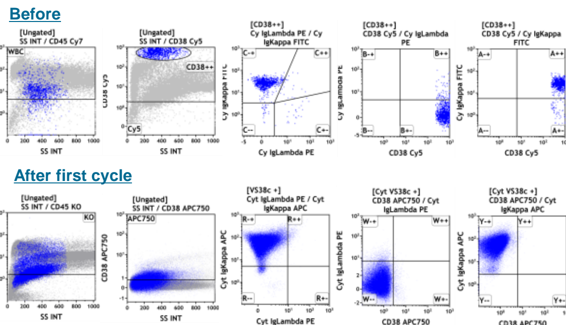
Pitfall: correct MoAb concentration but no signal CD38 in PCD: before and after treament



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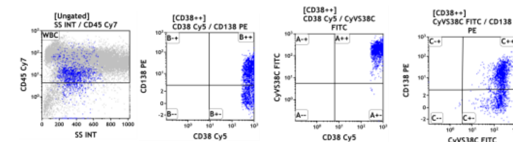
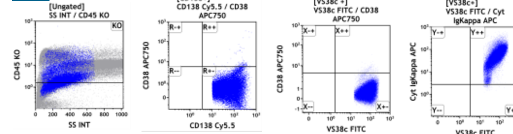
Before and after DaratumuMab





VS38c is still binding whereas CD38 is negative

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Treatment with Biologicals can be a nightmare in follow up of patients if you are not informed by the phycisian!

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- ***Bright Fluorochromes***
- Excellent (tandem) conjugates
- Pre-analytical prerequisites
- Highly sensitive Flow Cytometers
- Technical procedures

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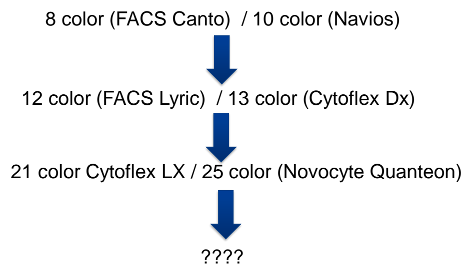
How many colors is enough: What is next?



8 color (FACS Canto) / 10 color (Navios)

12 color (FACS Lyric) / 13 color (Cytoflex Dx)

21 color Cytoflex LX / 25 color (Novocyte Quanteon)

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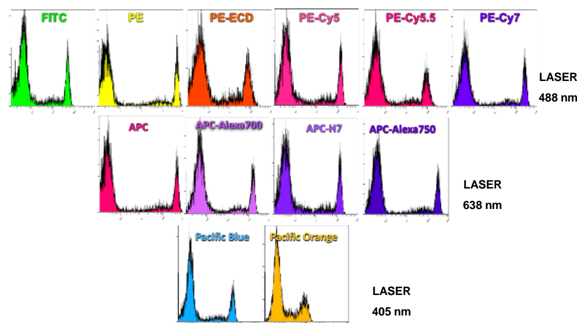
Fluorochromes available for 10-colours IPT

- Bright fluorochromes: to detect weak antigens

- But too bright means increase of spectral overlap



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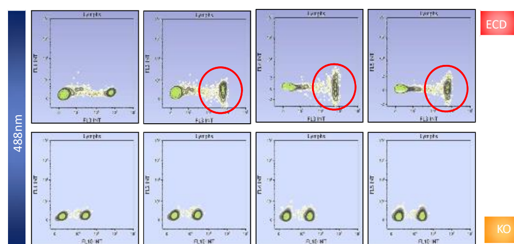
Data spread by Spillover



High expression is not always better! It can result in higher “spillover”, and localization of a part of cell population “under the axis”.

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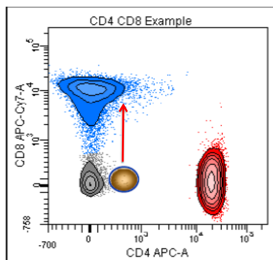
Data spread by Spillover

CD4dim and CD8neg -> identification OK
CD4dim and CD8pos -> identification problem!



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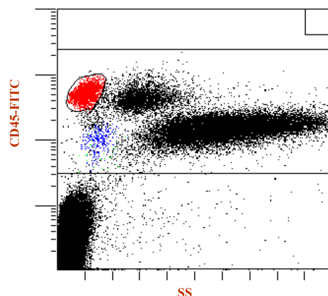
The use of CD45

with spillover in mind, what is the best conjugate



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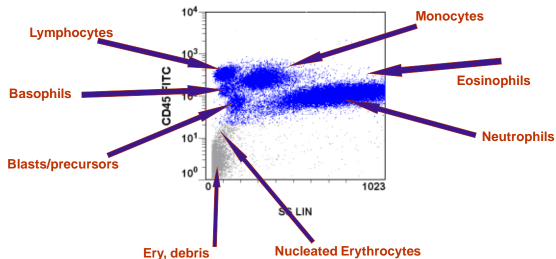
CD45: medium to high Ag density
CD45: available in most dyes

Cell population in CD45/SS plot of bone marrow



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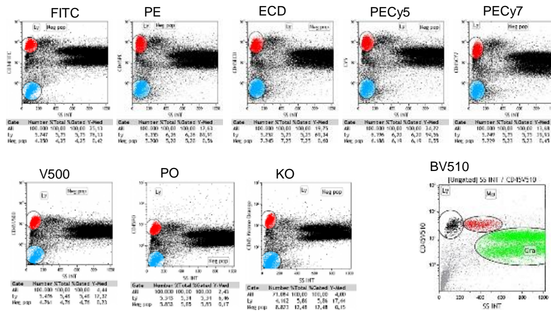
Mean Fluorescence intensities of CD45 conjugates



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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- ***Excellent (tandem) conjugates***
  - ***Improved conjugates***
  - ***Improved conjugation proces***
  - ***Stability***
- Pre-analytical prerequisites
- Highly sensitive Flow Cytometers
- Technical procedures

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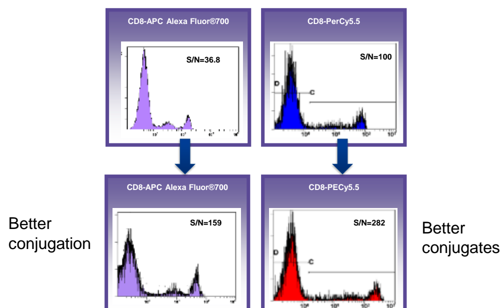
1. Improvement of conjugation / 2. Improvement of conjugates



Realize that batch to batch variation of conjugate occurs: This is not allowed in clinical FCM!!

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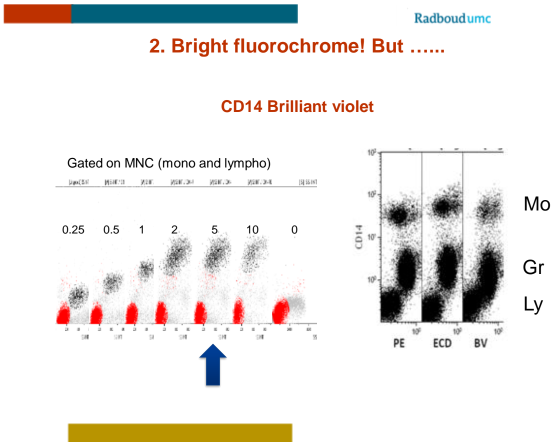
2. Bright fluorochrome! But ……

CD14 Brilliant violet



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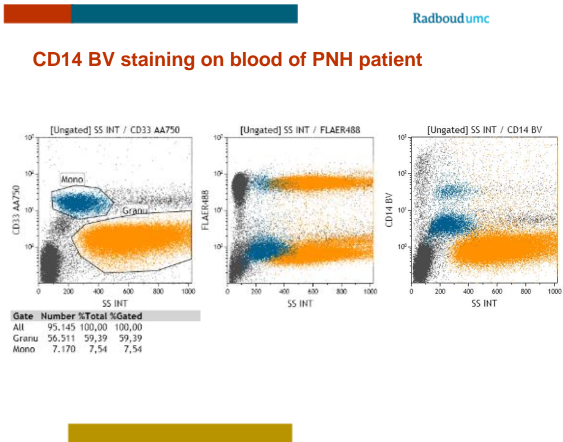
CD14 BV staining on blood of PNH patient



Gate	Number	%Total	%Gated
All	95.145	100,00	100,00
Granu	56.511	59,39	59,39
Mono	7.170	7,54	7,54

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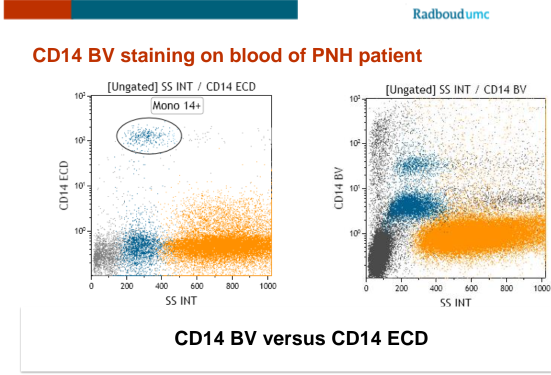
CD14 BV staining on blood of PNH patient



CD14 BV versus CD14 ECD

Investigate the right samples to draw your conclusion about a right or wrong conjugate!!

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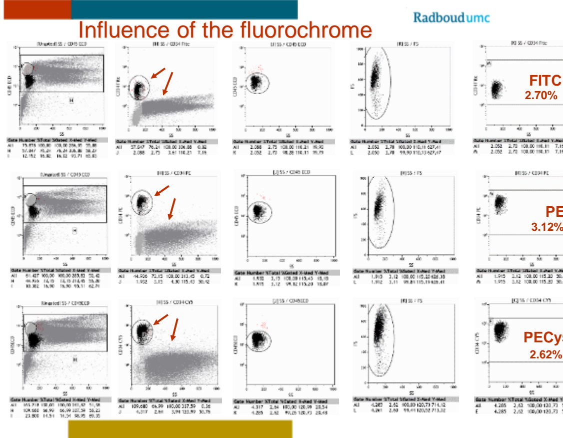
Influence of the fluorochrome



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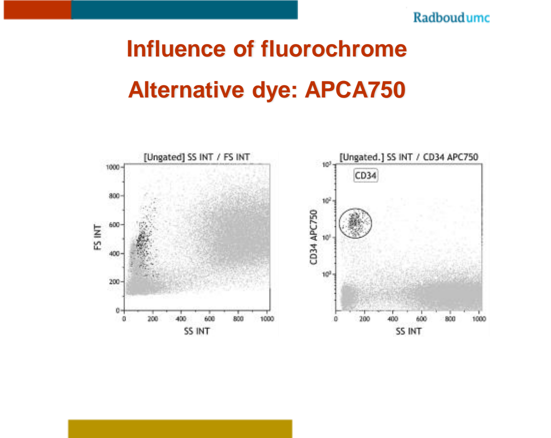
Influence of fluorochrome

Alternative dye: APCA750



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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- ***Excellent (tandem) conjugates***
  - ***Improved conjugates***
  - ***Improved conjugation proces***
  - ***Stability***
- Pre-analytical prerequisites
- Highly sensitive Flow Cytometers
- Technical procedures

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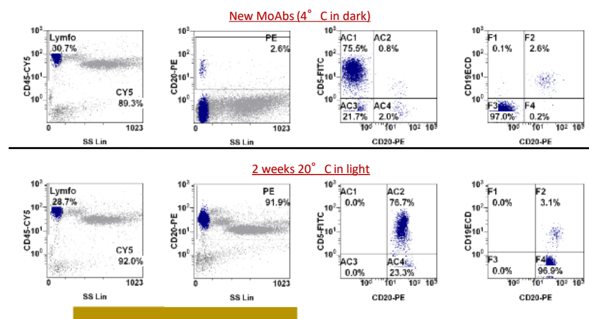
The importance of storage conditions

"What can happen if it does not matter you?"



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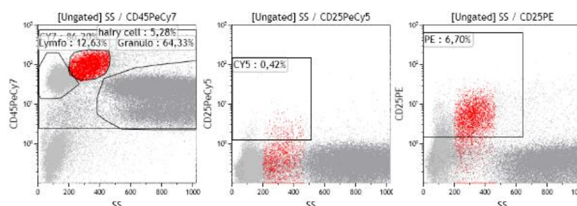
MoAbs in daily practice: check and check again. Does the conjugate work correctly?

Pitfall: CD25-PE versus CD25PE-CY5 in Hairy Cell Leukemia



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How to compose a multi-colour combination

- Which antigens should be determined?
- Clone selection - fluorochrome selection: availability and brightness
- (Which MoAb can be combined in the same colour: determine the specific and non-specific binding characteristics of the combined mAb conjugates)
- Single MoAb-conjugate titration
- Single MoAb-conjugate/SS determination: PMT settings
- Comparison of each MoAb in single MoAb expression
- Compensations of spectral overlap of each MoAb in the 10-colour panel (Fluorescence minus one [FMO])
- Analysis protocol (using KALUZA or INFINICYT)

FITC	PE	ECD	PECy5.5	PECy7	Pac B	Krome O	APC	APC-A700	APC-A750
CD34 Igkappa	CD7 Iglambda	CD4	CD10	CD117 CD56	CD15 CD20	CD45	CD3 CD33	CD8	CD19

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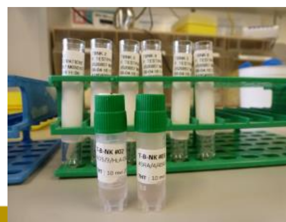
… And … Some open doors, but……

Use of MoAb panels:
- **Expiration of panels should be determined after mixing**
- **Use MoAbs that are within expiration during period of use of panels**
- **Panel filling out must be done programmed or by at least 2 persons**
- **Date of mixing and date of use should be archived**



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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Pre-analytical prerequisites (or headache)***
  - Sample collection and storage conditions
  - Incubation
  - Wash conditions
  - Lysing / permeabilization
- Highly sensitive Flow Cytometers
- Technical procedures

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Items in the pre-analytical FCM-protocol that cause errors

- Cellularity: FNA, CSF, hypoplastic BM
- Fragility of cells: DLBCL
- Necrosis: Morphology may confirm
- Cell loss: Plasma cell loss by filtration of BM
- Disassociation of abnormal cells: Lymph node, tissues
- Autolysis, debris: Age, storage processing
- Antigen loss: Ph, time, fixation, using of target antibodies >Room Temp.

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Pre-analytical prerequisites***
  - ***Sample collection and storage conditions***
  - ***Incubation***
  - ***Wash conditions***
  - ***Lysing / permeabilization***
- Highly sensitive Flow Cytometers
- Technical procedures

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Items in the pre-analytical FCM-protocol that cause errors

- Errors in drawing of blood: Infuence on cell populations by
  - Condition of the patient
  - Stowage
  - Anticoagulation: No, EDTA / Heparin / ACD
- Errors in bone marrow aspiration:
  - Dry tap
  - Sampling error (first collection for smears, second for IPT
  - Anticoagulation: Heparin, ACD+heparin
  - Blood contamination
    - Calculate the % blood in bone marrow by Holdrinet formula

    (% = WBC-BL x RBC-BM / WBC-BM x RBC-BL)
- Transport: duration, temperature
- Storage: medium (CSF), temperature

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Pre-analytical prerequisites***
  - ***Sample collection and storage conditions***
  - ***Incubation***   ***- Cell concentration***
    - Volume vs. MoAb concentation
    - Sample age
    - Viability staining
  - ***Wash conditions***
  - ***Lysing / permeabilization***
- Highly sensitive Flow Cytometers
- Technical procedures

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Which factor is critical?

Variation of cell number: antigen concentration

		1.000.000 cells			100.000 cells			10.000 cells		
		Pos. Population MFI	Neg. Population MFI	Ratio	Pos. Population MFI	Neg. Population MFI	Ratio	Pos. Population MFI	Neg. Population MFI	Ratio
Ig Kappa	FITC	4,56	0,59	8	4,81	0,68	7			
CD34	FITC	9,53	0,4	24	10,17	0,43	24	9,78	0,38	26
Ig Lambda	PE	16,06	0,65	25	18,21	0,63	29			
CD7	PE	10,32	0,31	33	11,10	0,33	34	10,64	0,4	27
CD4	Cy5.5	12,98	0,28	46	14,03	0,3	47	12,47	0,26	48
CD117	Cy7	18,99	0,75	25	19,37	0,82	24	19,48	0,68	29
CD3	APC	20,24	0,82	25	21,11	1,02	21	18,23	0,97	19
CD33	APC	62,03	0,41	151	64,72	0,76	85	61,55	2,00	31
CD8	APC A700	70,45	0,43	164	77,63	0,51	152	67,10	0,44	153
CD19	APC A750	8,24	0,10	82	8,29	0,10	83	8,81	0,10	88
CD15	PB	14,71	0,13	113	16,68	0,14	119	10,28	0,12	86
CD20	PB	14,41	0,28	51	13,17	0,29	45	13,97	0,29	48

1. # of cells is not critical: S/N is comparable by various numbers of cells

   (Ag-Ab ratio is not relevant due to the high affinity MoAbs)

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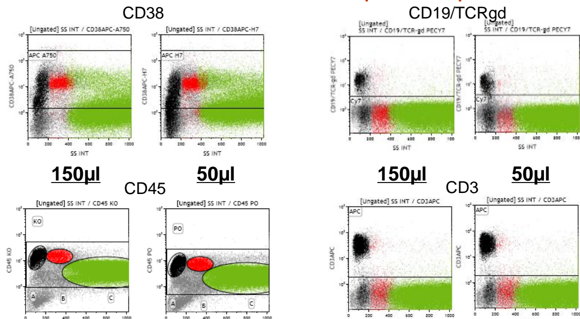
Variation of Incubation: volume 150µl versus 50µl



1. # of cells is not critical: S/N is comparable by various # cell
2. Volume is not critical
3. **But concentration of MoAb is critical (See the titrations!)**

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Pre-analytical prerequisites***
  - ***Sample collection and storage conditions***
  - ***Incubation***   ***- Volume vs. concentration***
    - Sample age
    - Viability staining
  - ***Wash conditions***
  - ***Lysing / permeabilization***
- Highly sensitive Flow Cytometers
- Technical procedures

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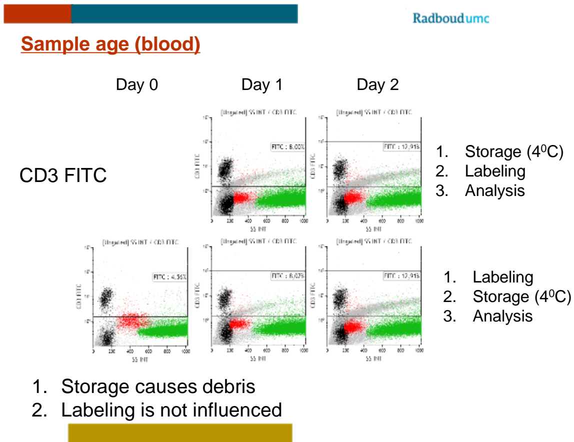

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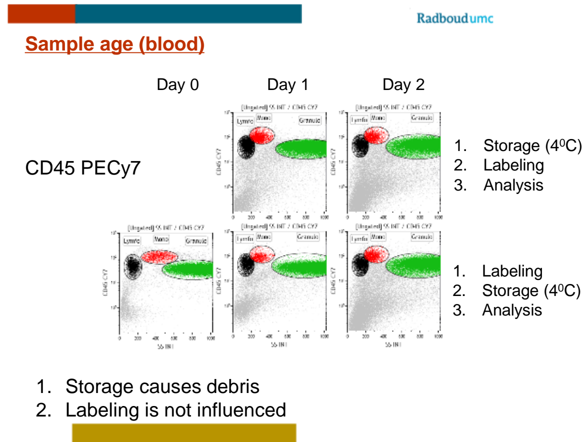

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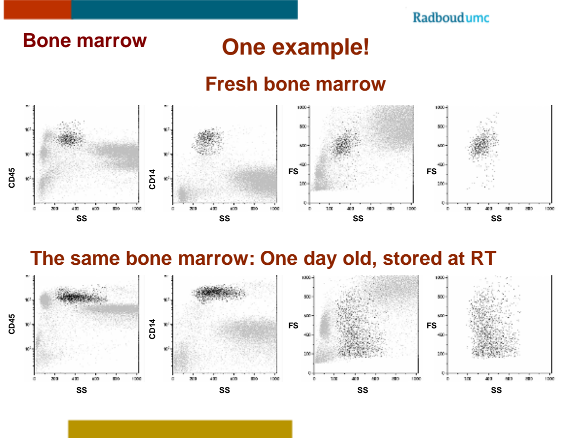

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Incubation prerequisites***
  - ***Sample collection and storage conditions***
  - ***Incubation*** - ***Volume vs. concentation***
    - ***Sample age***
    - ***Viability staining***
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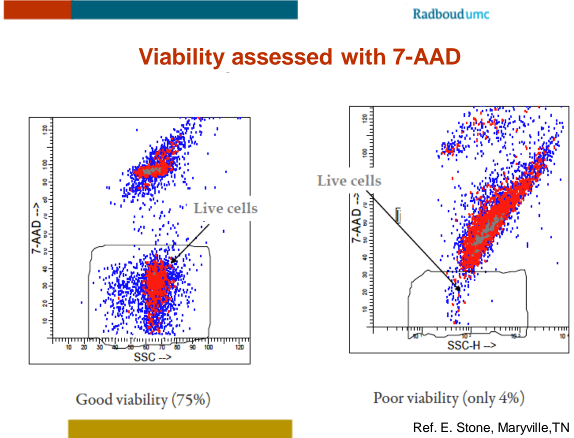

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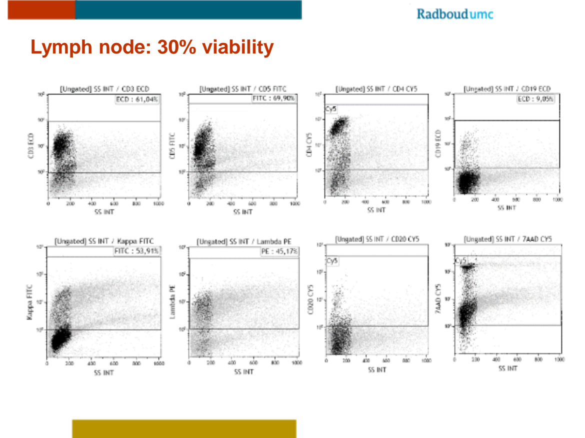

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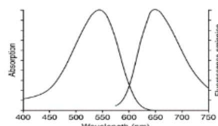
DNA-Intercalation dye (7-AAD)

- 7-Aminoactinomycine D (7-AAD) $C_{62}H_{87}N_{13}O_{16}$
  - is currently used as a standard DNA intercalating dye to determine the amount of dead and early apoptotic SCs by FCM.
- Spectral properties:
  - Excitation max. at 546 nm
  - Emission max. at 647 nm



Wikipedia.org

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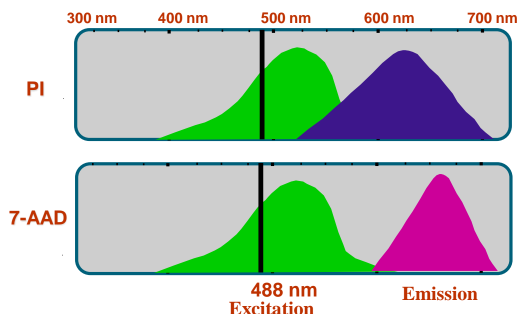
Propidium Iodide versus 7-AAD



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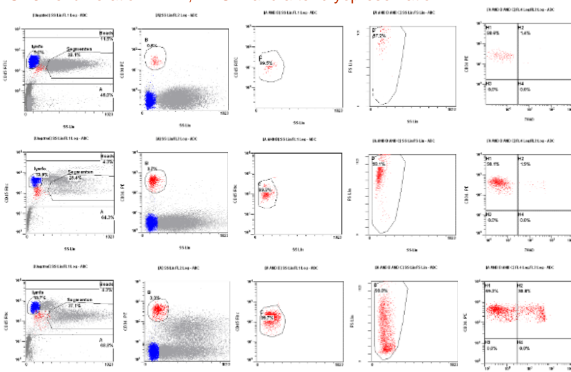
CD34 enumeration in PB, HPC-A and after cryopreservation



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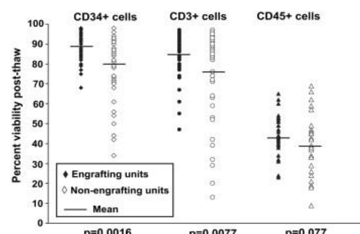
Low CD34+ Cell Viability Have a Low Probability of Engraftment after Double Cord Blood Units

Units with <75% viability are very unlikely to engraft



Scaradavou et al.
Transplantation Biology of Blood and Marrow Transplantation 16, 4 :500-508, 2010

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Dead / viable staining dyes

- **DNA binding dyes**
  - PI, 7-AAD (Old and Long history: 7-AAD and PI)
  - Sytos dye, DRAQ7: Easy to use at end of staining
  - Not all can be used with fix/perm procedures
- **New Amine dyes**
  - ViaKrome, Zombie dyes, ViVid, Aqua blue, etc.
  - Impermeable but binding of amine groups: Live cells possess a few amines outside the cells (dim staining), dead cells much more (bright staining)
  - Easy to use in fixed cells (dyes are fixable)
  - Dyes are expensive
- **Vital dyes**
  - DRAQ5, Hoechst, etc.
  - Staining of viable cells
  - Easy to use

Conclusion: DNA binding dyes seems to be OK but the new Amines are better

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Viability staining: exclusion of dead cells AND apoptotic cells

7-AAD stains dead cells but not (not-effectively/insufficiently) apoptotic cells

Besides, 7-AAD can non-specifically (!) bind to cells resulting in an increase of non-viable cells

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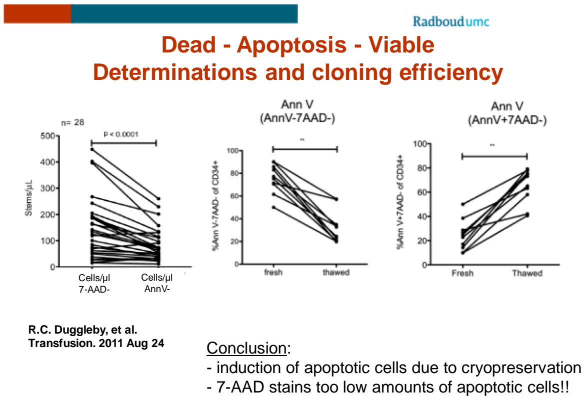

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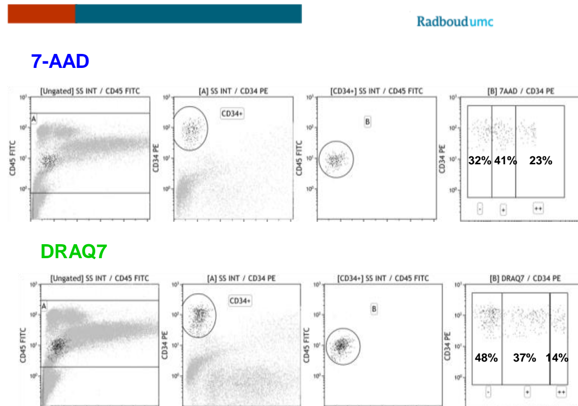

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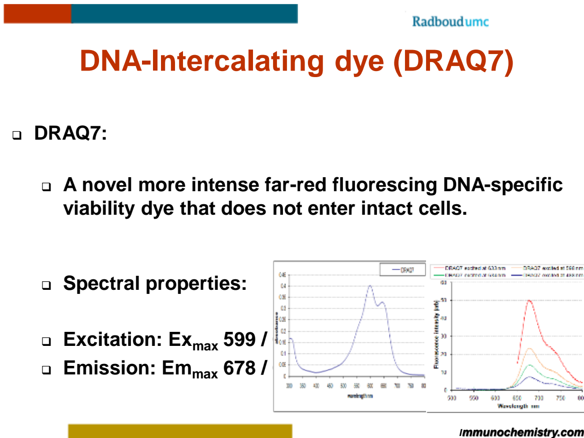

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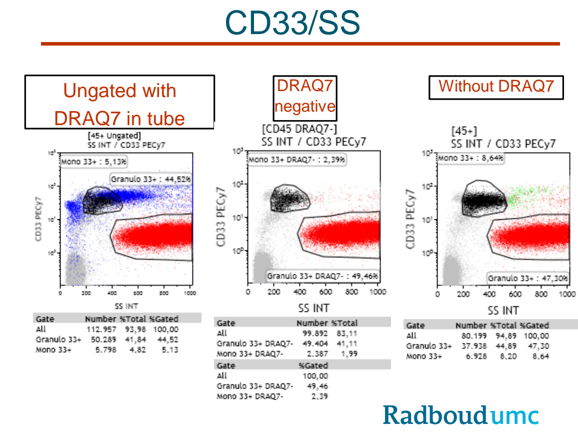

Gate	Number	%Total	%Gated
All	112.957	93,98	100,00
Granulo 33+	50.289	41,84	44,52
Mono 33+	5.798	4,82	5,13

Gate	Number	%Total
All	99.892	83,11
Granulo 33+ DRAQ7-	49.404	41,11
Mono 33+ DRAQ7-	2.387	1,99

Gate	%Gated
All	100,00
Granulo 33+ DRAQ7-	49,46
Mono 33+ DRAQ7-	2,39

Gate	Number	%Total	%Gated
All	80.199	94,89	100,00
Granulo 33+	37.938	44,89	47,30
Mono 33+	6.928	8,20	8,64

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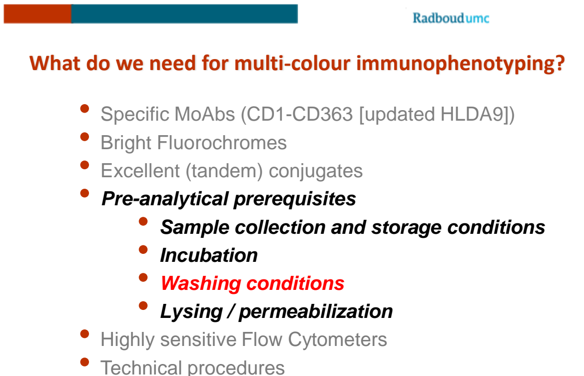

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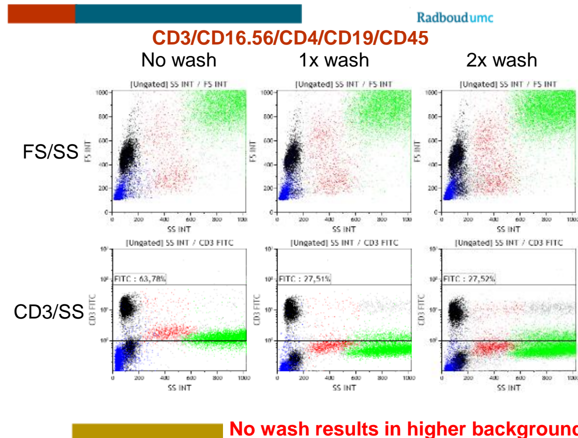

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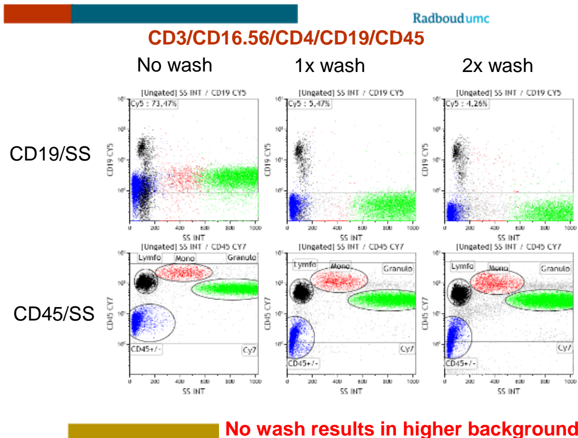

No wash results in higher background

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What do we need for multi-colour immunophenotyping?

- Specific MoAbs (CD1-CD363 [updated HLDA9])
- Bright Fluorochromes
- Excellent (tandem) conjugates
- ***Pre-analytical prerequisites***
  - ***Sample collection and storage conditions***
  - ***Incubation***
  - ***Wash conditions***
  - ***Lysing / permeabilization***
- Highly sensitive Flow Cytometers
- Technical procedures

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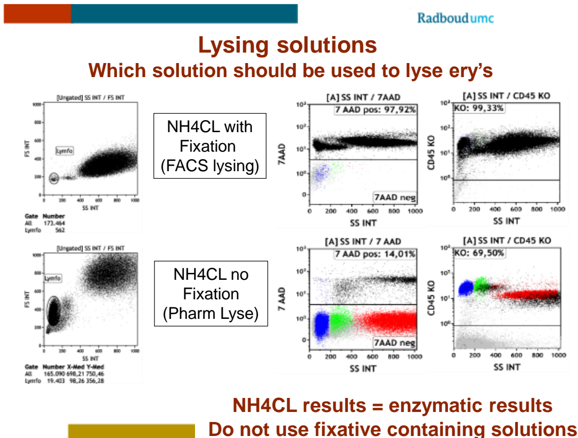
Lysing solutions

Which solution should be used to lyse ery's



NH4CL results = enzymatic results
Do not use fixative containing solutions

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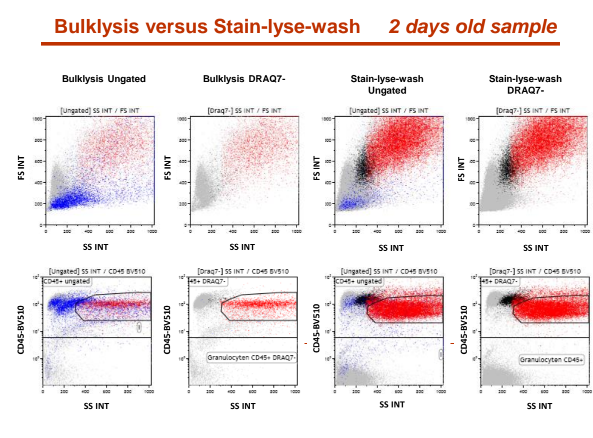
Bulklysis versus Stain-lyse-wash ***2 days old sample***



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Determination of erythrocyte precursors in MDS

Influence of

(a) Lysing procedure
Bulk lysis (NH4CL based) versus unlysed

(b) Dead cells
Exclusion of dead by 7-AAD and DRAQ7

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The panels used to answer the question

	FITC	PE	ECD	PC5	PC7	APC	AA700	AA750	BV421	BV510
1	CD36	CD235a	CD45	7AAD	CD117	CD34	DRAQ7	CD71	CD33	
2	CD71	CD105		7AAD	CD117	CD36	DRAQ5		CD33	CD45

Why these tubes:

1. **Lysis of cells in the dead-cell exclusion tube**
2. **Unlysed tube (CD235a is not possible due to binding to ery's)**

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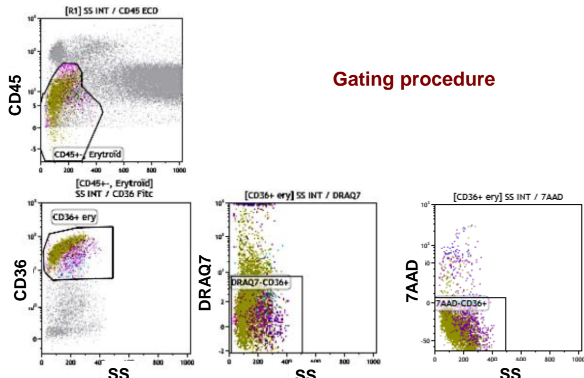
	FITC	PE	ECD	PC5	PC7	APC	AA700	AA750	BV421
1	CD36	CD235a	CD45	7AAD	CD117	CD34	DRAQ7	CD71	CD33



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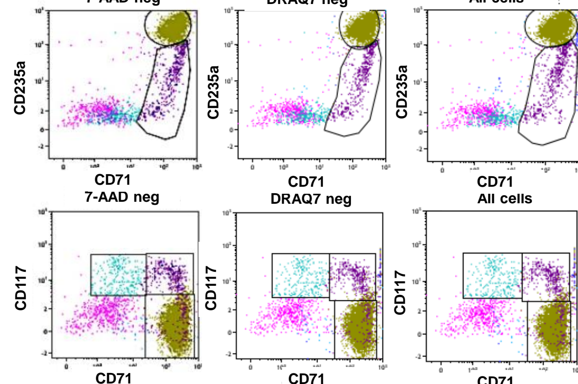
	FITC	PE	ECD	PC5	PC7	APC	AA700	AA750	BV421
1	CD36	CD235a	CD45	7AAD	CD117	CD34	DRAQ7	CD71	CD33



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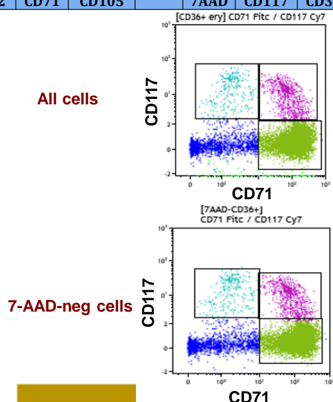
	FITC	PE	ECD	PC5	PC7	APC	AA700	BV421	BV510
2	CD71	CD105		7AAD	CD117	CD36	DRAQ5	CD33	CD45



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Influence of dead cells and lysis

Results of the mean of 10 independent determinations

	Bulk Lysis	CD117+ CD71+	CD117+ CD71-	CD117- CD71+	CD235a+ CD71+	CD235a+-CD71+
Tube 1	DRAQ7 - neg	0,17	0,05	1,81	1,77	0,21
	7-AAD - neg	0,17	0,06	2,08	2,05	0,21
	All cells	0,22	0,06	2,10	2,10	0,26

CD117+ CD105+	CD117+ CD105-	CD117- CD105+	CD117+ CD71+	CD117+ CD71-	CD117- CD71+	No lysis	
0,19	0,07	0,44	0,43	0,05	4,87	7-AAD - neg	*Tube 2*
0,23	0,08	0,45	0,40	0,06	5,10	All cells	

What is clear

1. Difference between 7-AAD and DRAQ7
2. Difference between lysis and no lysis
3. Difference between viable cells and all cells

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Summary of pre-analyses conditions

- Use selected MoAb conjugates
- Use washed sample: free Moabs and plasma contains Ig!
- Titrate MoAbs in single **and** in balanced panels to determine correct concentration based on S/N
- Cells must be in homogeneous suspension
- Incubate 15 min at 4-8^0 C in complete dark
- Use PBS + HSA/BSA to avoid non-specificity

Keep in mind, than you start the FCM analysis…..

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Thanks for your attention

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