ERN-EuroBloodNet Topic on Focus on Inherited Platelet Function Disorders (IPFD)

webinar

HEALTH PROFESSIONALS

Flow cytometry for IPFD – advantages and limits

Sofia Ramström, PhD, Associate professor
School of Medical Sciences, Cardiovascular Research Centre, Örebro University
ISTH SSC Platelets in Health and Disease chair

January 21, 2026

CRPP Centre de Référence Pathologies Plaquettaires
MHEMO La Filière des maladies rares de l'hémostase
ebn ERN | EuroBloodNet

Disclosure of conflicts of interest

No conflicts of interest related to the content of this lecture.

Inherited Platelet Function Defects

Patient presenting with bleeding tendency

→ Are platelet functions affected?

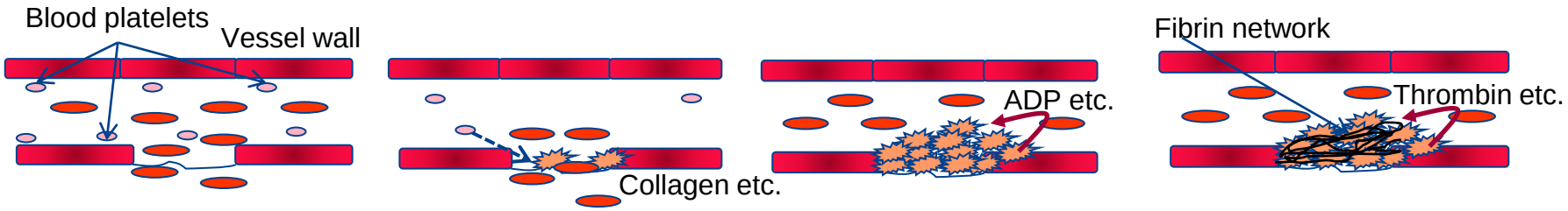
→ Which platelet functions are affected?

Genetic defect identified

→ will it cause clinically relevant functional defects?

→ will it cause bleeding/thrombotic problems?

Many platelet functions are relevant for haemostasis…



Vessel wall damage

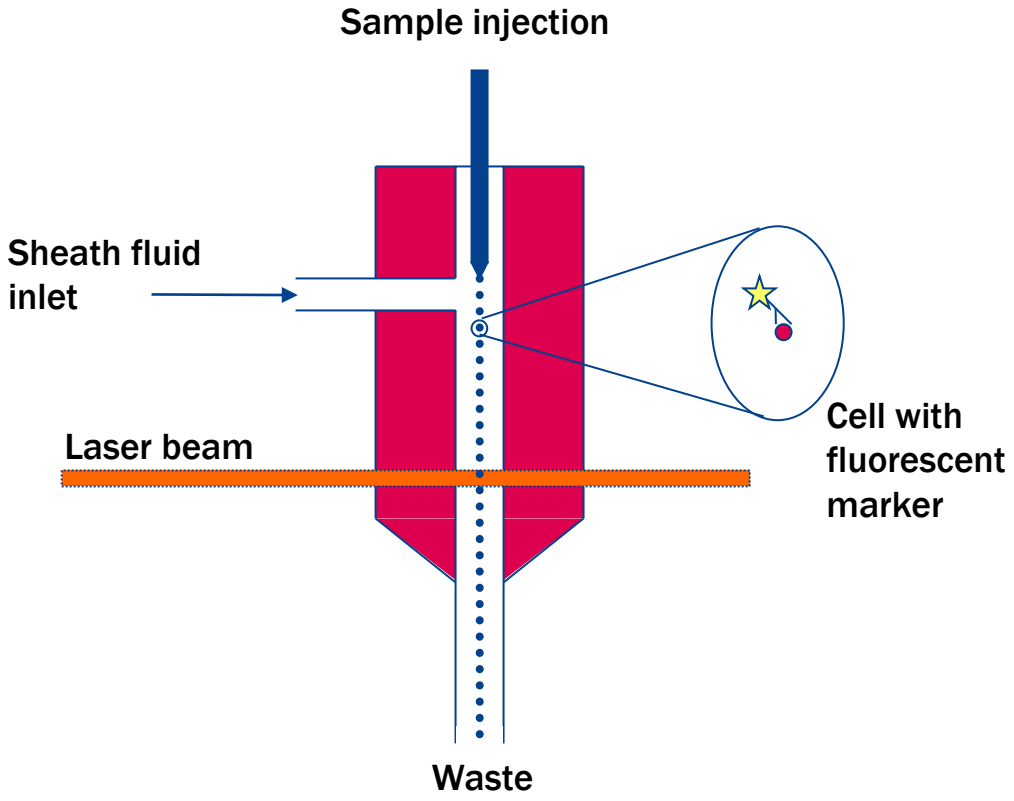
Adhesion

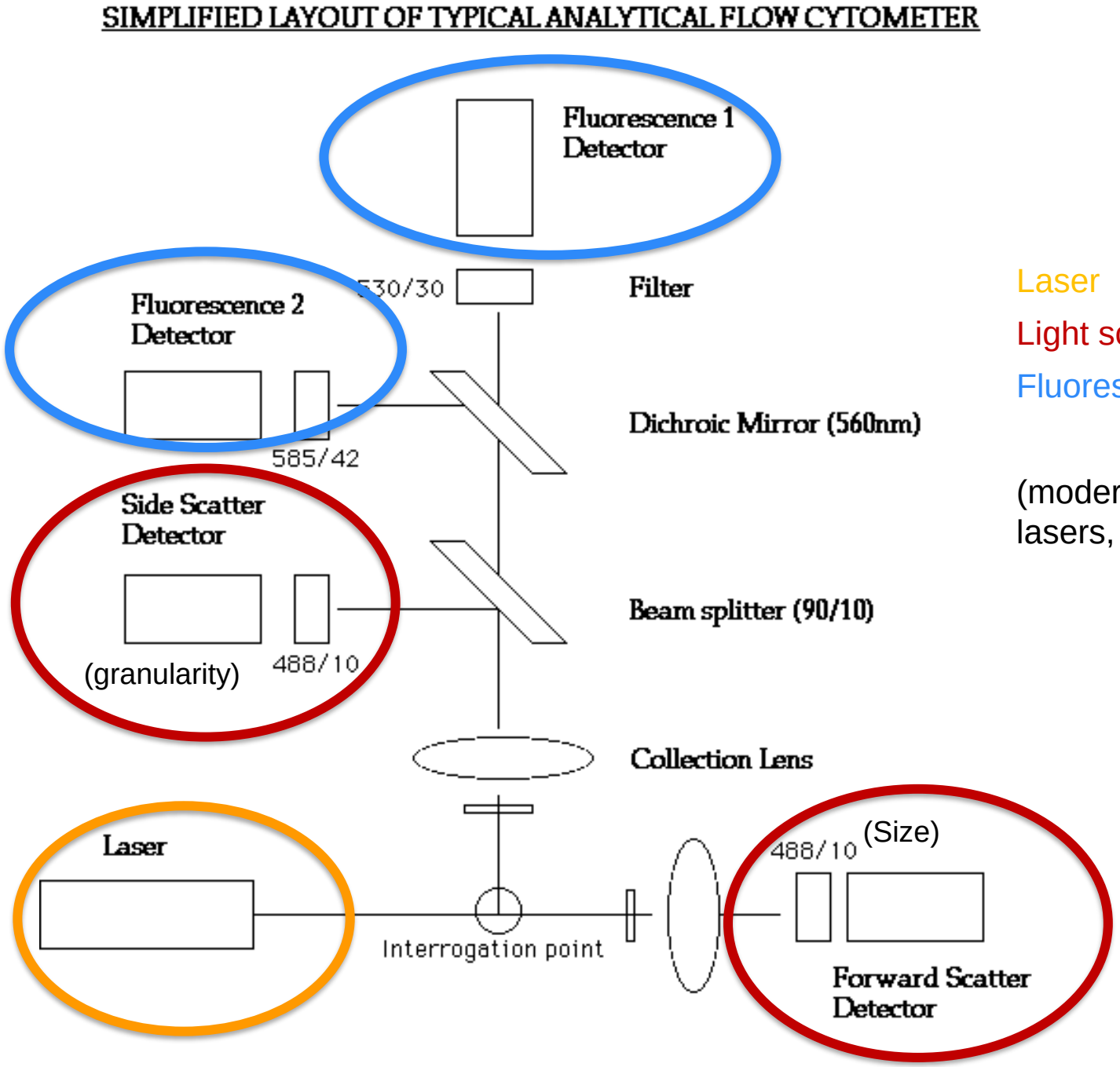
Fibrinogen receptor activation → aggregation
Granule release (alpha, dense, lysosomal)

Surface membrane changes → release of microparticles, catalysation of coagulation

…but many methods only measure one of these (e.g aggregation, secretion assays, thrombin generation)
→ advantage for flow cytometry

Introduction to flow cytometry





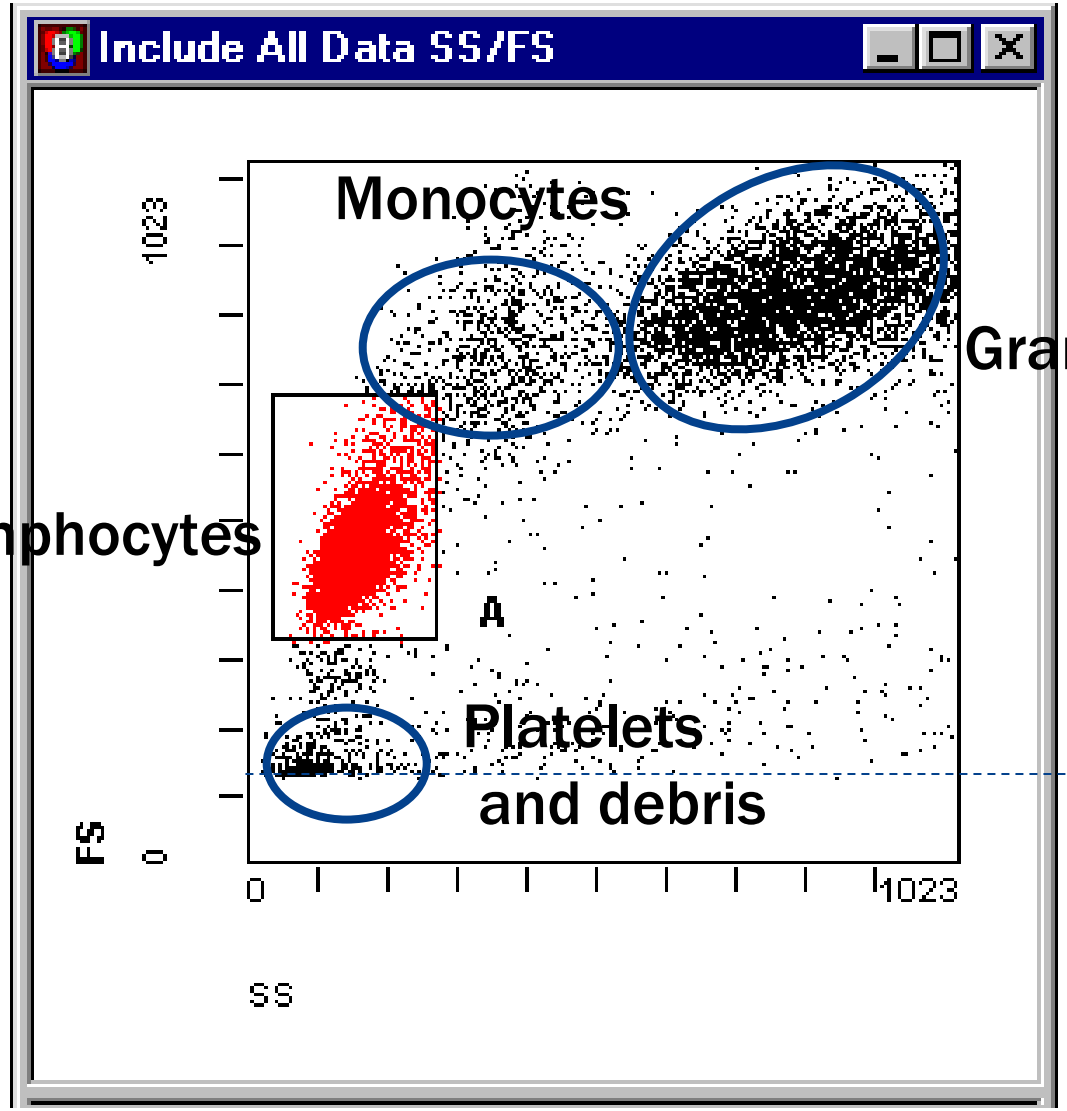
Laser

Light scatter detectors

Fluorescence detectors

(modern instruments: more lasers, more detectors…)

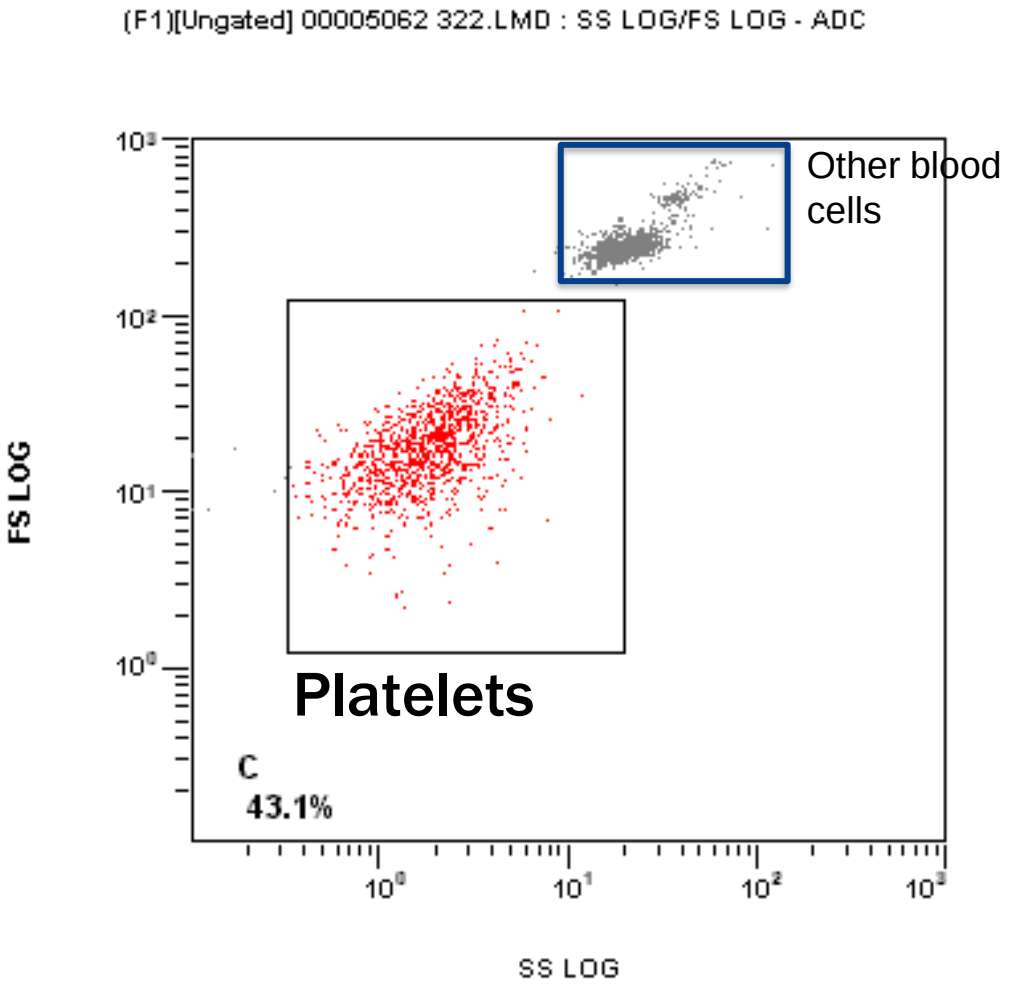
Flow cytometry – where are the platelets..?



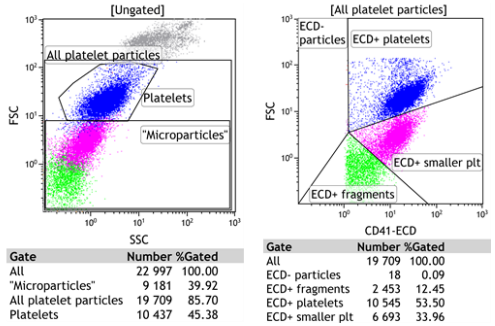
Lysed whole blood, FSC vs. SSC linear scale:

Discriminator (threshold)– Now cutting out our particles of interest…

The size advantage



- No lysis needed, whole blood can be used
- No risk of loosing platelet populations during centrifugation



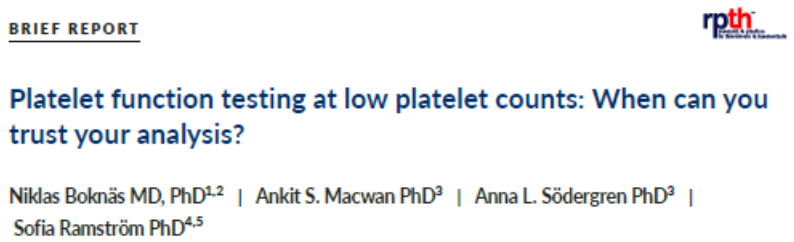
With threshold on fluorescence → smaller ”microparticles” may also be detected

Clean instrument? – check the washing protocols!

Avoid RBC lysis in non-fixed samples (ADP release)

Advantages with flow cytometry for platelet function testing

- Whole blood can be used
- Platelet pre-analytical activation can be easily tested and accounted for
- Low volumes of blood are needed
- Platelet function can be evaluated also in thrombocytopenic patients

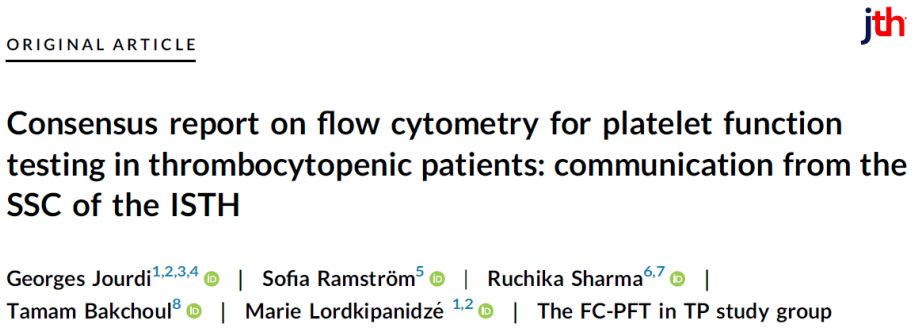


BRIEF REPORT

rpth

Platelet function testing at low platelet counts: When can you trust your analysis?

Niklas Boknäs MD, PhD[1,2] | Ankit S. Macwan PhD[3] | Anna L. Södergren PhD[3] | Sofia Ramström PhD[4,5]



ORIGINAL ARTICLE

jth

Consensus report on flow cytometry for platelet function testing in thrombocytopenic patients: communication from the SSC of the ISTH

Georges Jourdi[1,2,3,4] | Sofia Ramström[5] | Ruchika Sharma[6,7] | Tamam Bakchoul[8] | Marie Lordkipanidzé[1,2] | The FC-PFT in TP study group

Advantages with flow cytometry for platelet function testing

- Tests can include many different aspects of platelet function
- Surface expression of important receptors can be evaluated at the same time

Advantages with flow cytometry for platelet function testing

Table 1 (Continued)

First-step laboratory tests			
Test	**Parameter**	**Result**	**IPFD**
	Arachidonic acid	Altered aggregation	COX-1[31] TP defect[32] GT[21] $P2Y_{12}$ defect[27] δ-SPD[24] FPD/AML/MDS[23] ARC (±)[8] White platelet syndrome (±)[13]
	Ristocetin	Altered aggregation	PT-VWD[5] BSS[2] GATA1[9]
Granule release (luminometry, ELISA)	α granules	Defective release	GPS[3] QPD (protein degradation)[19] WAS (±)[1,22] ARC[8] Stormorken syndrome[15] GATA1[9]
	δ granules	Defective release	δ- SPD[24] HPS[16] CHS[16] WAS (±)[1,22] FPD/AML/MDS[23] Filaminopathy (±)[33]
	α-δ granules	Defective release	PSD[24,25] α-δ SPD[34]
Flow cytometry (screening)	GPIIb/IIIa GPIIIa GPIb/IX	Defective expression Defective expression Defective expression	GT[21] GT[21] BSS[2] VCF[7]
	GPIb GPIIb/IIIa activation (PAC-1)	Defective expression Defective activation	BSS[2] GT[21] Stormorken syndrome[35] PKC defects[36,37]

Flow cytometry is already suggested as part of first line investigations, so why not use it for more tests…?

From: Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. Gresele P, for the Subcommittee on Platelet Physiology. *J Thromb Haemost* 2015; 13: 314–22.

Challenges with flow cytometry for platelet function testing in clinical settings

- Fresh blood is necessary
- Need for a skilled operator and a lot of pipetting…
- Flow cytometers are expensive instruments (but used for many different things, so often present in hospitals)
- A lot of data is generated
- Correct data analysis is crucial for reliable interpretation

Possible solutions

- Pre-frozen reagent mixtures with antibodies and/or platelet activators
- Standardized protocols for preparation of samples and running the flow cytometer

→ possible for less experienced users to prepare and run samples for later (also remote) data analysis

Platelet agonists

Platelet agonist	Receptors	Remarks
Thrombin	PAR1, PAR4, GPIb/V/IX	GPRP peptide needed to prevent fibrin polymerization in PRP/whole blood samples. Do not use with anticoagulants that inhibit thrombin
PAR1-AP	PAR1	The most commonly used and potent peptide has amino acid sequence SFLLRN.[116] Also called "TRAP" or "TRAP-6." SFLLRN also activates PAR2,[117] but this receptor is not present on platelets[118]
PAR4-AP	PAR4	The most used and most active peptide has amino acid sequence AYPGKF, and is based on the mouse PAR4 tethered ligand[119]
ADP	$P2Y_1$, $P2Y_{12}$	Autologous ADP also contributes to the response of other platelet agonists (see ►**Fig. 1G–I**)
CRP-XL	GPVI	Cross-linking is needed for CRP to be active.[120] Fibrillar type I ("Horm") collagen is not an effective agonist in flow cytometry, although methylated acid-soluble collagen type I is more potent[121]
Convulxin	GPVI	C-type lectin isolated from *Crotalus durissus terrificus* snake venom[122]
Epinephrine (adrenaline)	α_2A-adrenergic receptor	Usually only potentiating effects in washed platelet suspensions, but direct effects in PRP and whole blood[123]
U46619[124]	TXA_2 receptor (TP)	PGH_2 analog, potent and stable TP receptor agonist[125]
Rhodocytin,[126] fucoidan[127]	CLEC-2	Podoplanin is the physiological ligand for CLEC-2[128]
Serotonin	5-HT_{2A}	Reported to enhance activation by other agonists in whole blood assays[129]
Arachidonic acid	TP (after conversion to TXA_2 by COX-1 and thromboxane synthetase)	Problems with cell lysis reported, which needs to be checked and proven not to influence the results (Ca^{2+} and plasma may be somewhat protective)[26,130–134]
Ristocetin	Induces conformational changes allowing VWF to bind to GPIb	Has been used to design flow cytometry–based tests for von Willebrand disease.[135] However, problems with cell fragmentation[26] and platelet apoptosis[136] have been reported

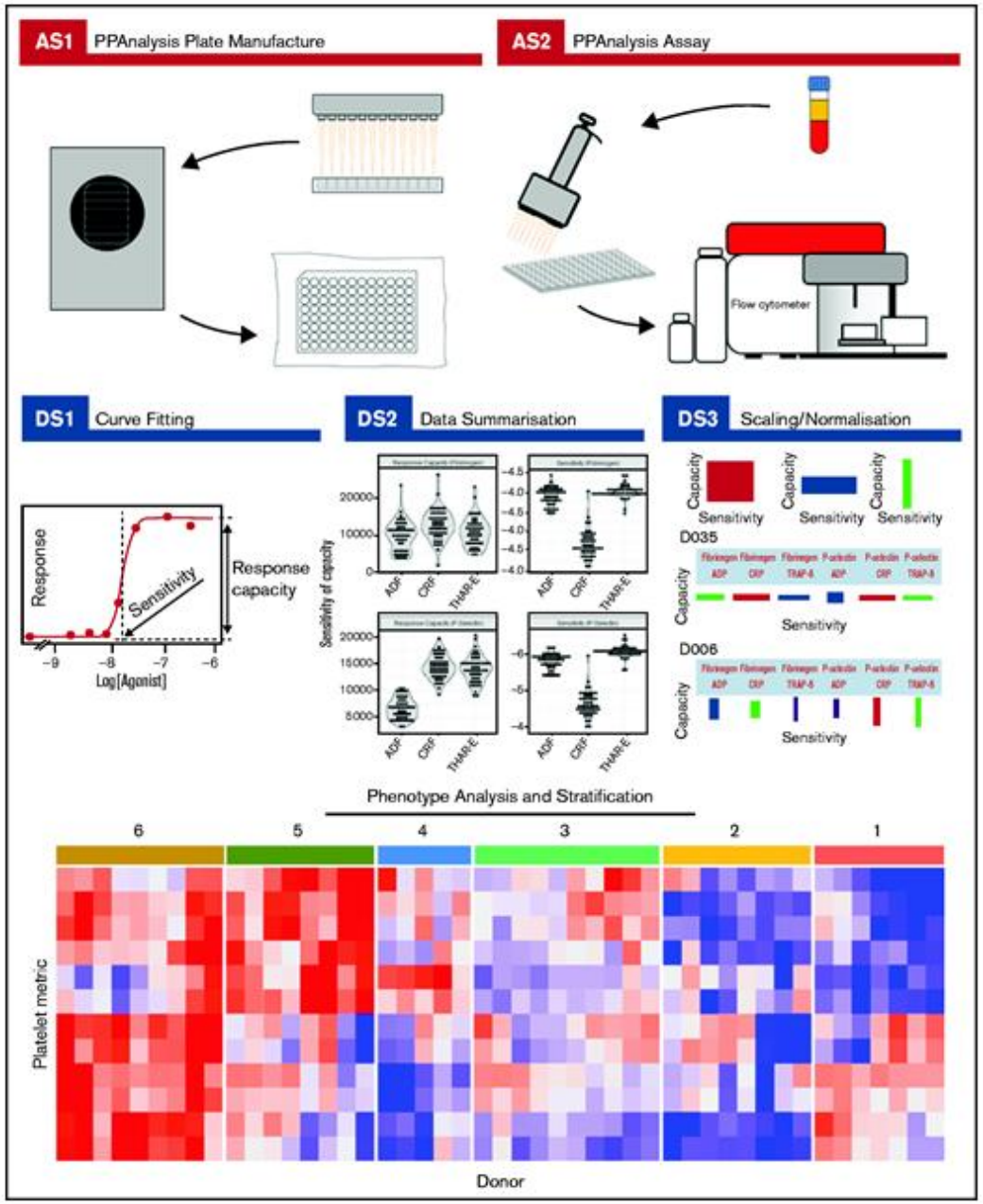
Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

Challenge – defects are most obvious at low grade activation, but the inter-individual variation is also greater here
→ harder to tell what is a defect

Possible solution: to evaluate concentration-response curves?

Platelet agonists - evaluating concentration-response curves

“Sensitivity and capacity are distinct characteristics of platelet function”



Dunster et al. Multiparameter phenotyping of platelet reactivity for stratification of human cohorts. Blood Adv. 2021 Oct 26;5(20):4017-4030.

Platelet functions

Activation event	Marker(s) with references	Other names, remarks	Possible negative control(s)
Fibrinogen receptor GPIIb/IIIa activation	PAC-1,[29] LIBS/RIBS-specific antibodies,[58–63] fluorescent fibrinogen,[64,65] anti-fibrinogen antibodies[8]	GPIIb/IIIa = $\alpha IIb\beta_3$, CD41/61	EDTA-treated platelets, isotype control
Collagen receptor GPIa/IIa activation	Antibody detecting conformational change[66]	GPIa/IIa = $\alpha_2\beta_1$, CD49/CD29	Isotype control
Alpha granule exocytosis	P-selectin,[67–69] CD40L,[70] thrombospondin[71,72]/GMP-33,[73,74] multimerin[75,76]	P-selectin = CD62P, GMP140, PADGEM	Isotype control
		CD40L = CD154	
		GMP-33 = N-terminal fragment of thrombospondin[73]	
Dense granule exocytosis	Mepacrine staining,[77–79] *CD63*,[80] *LAMP-2*[81,82]	CD63 = LIMP, LA… granulophysin[83]	Nonlabeled platelets (mepacrine), isotype control
		LAMP-2 = CD107b	
Lysosomal exocytosis	LAMP-1,[84,85] *LAMP-2*,[81,82] *CD63*[80]	LAMP-1 = CD107a	Isotype control
PS exposure	Annexin V,[39–41,86] lactadherin[87–89]	Ca^{2+} is required for annexin V binding	Sample without calcium (annexin V)/resting platelets (lactadherin)
Coagulation factor binding	Factor V/Va,[90,91] X/Xa,[92] VIII[93]	Ca^{2+} is required for coagulation factor complex formation[42]	Isotype control
Platelet vesiculation (microparticle formation)	Antibodies toward surface receptors (e.g., GPIIb, GPIIIa, GPIb, GPIX) or annexin V/lactadherin[53] in combination with size characteristics	Better detected with threshold on fluorescence instead of scatter	Resting platelets
Intracellular phosphorylation events	VASP[94]	Designed to measure the function of the platelet ADP receptor $P2Y_{12}$ (more discussed below)	
Changes in intracellular calcium levels	Fluo-3,[95] Fluo-4,[96] Indo-1[97]	Ratiometric measurements are possible by combining dyes with Fura Red[96]	Resting platelets
Changes in mitochondrial membrane potential	A variety of probes are available, based on, for example, carbocyanine (e.g., JC-1), rhodamine (e.g., TMRM) and rosamine (e.g., CMXRos).[98,99]		Resting platelets (for intact membrane), FCCP/CCCP-treated platelets (for fully depolarized membrane)[98,100]

Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

Granule content/release analysis - challenges

- Proof of content is not proof of release
- Proof of exocytosis is not proof of functional content

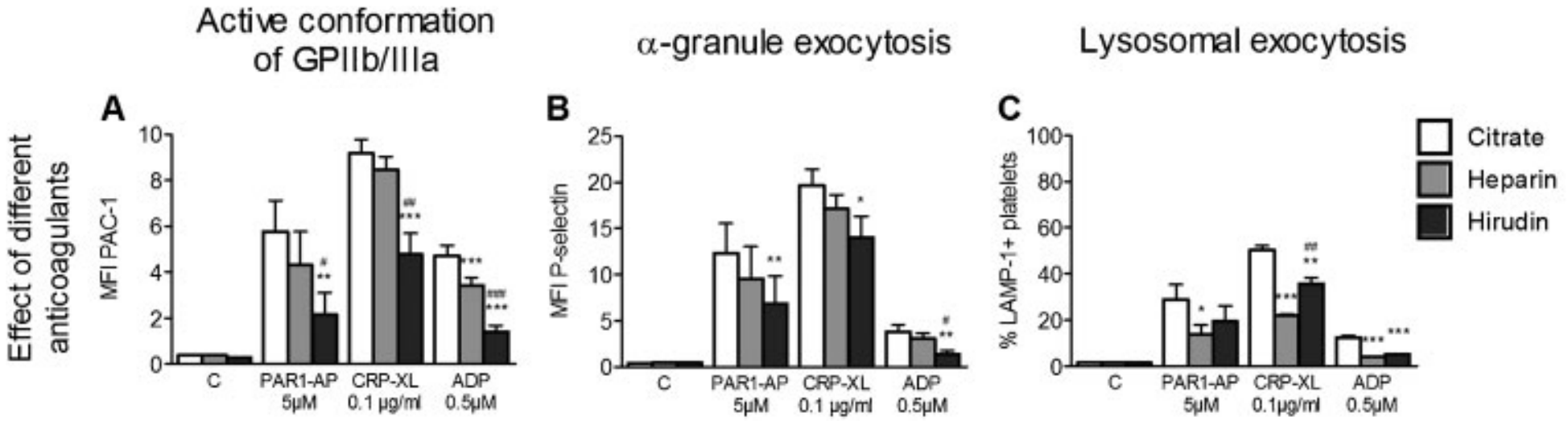
- Note: most markers are not totally specific to one type of granule!

ISTH SSC Platelets in Health and Disease

Current Projects

- International multicenter assessment of methods to detect platelet dense granule deficiencies
  project began 2022, projected end TBD
- How do soluble platelet-derived proteins relate to platelet reactivity in cardiovascular patients?,
  project began 2024, projected end 2027

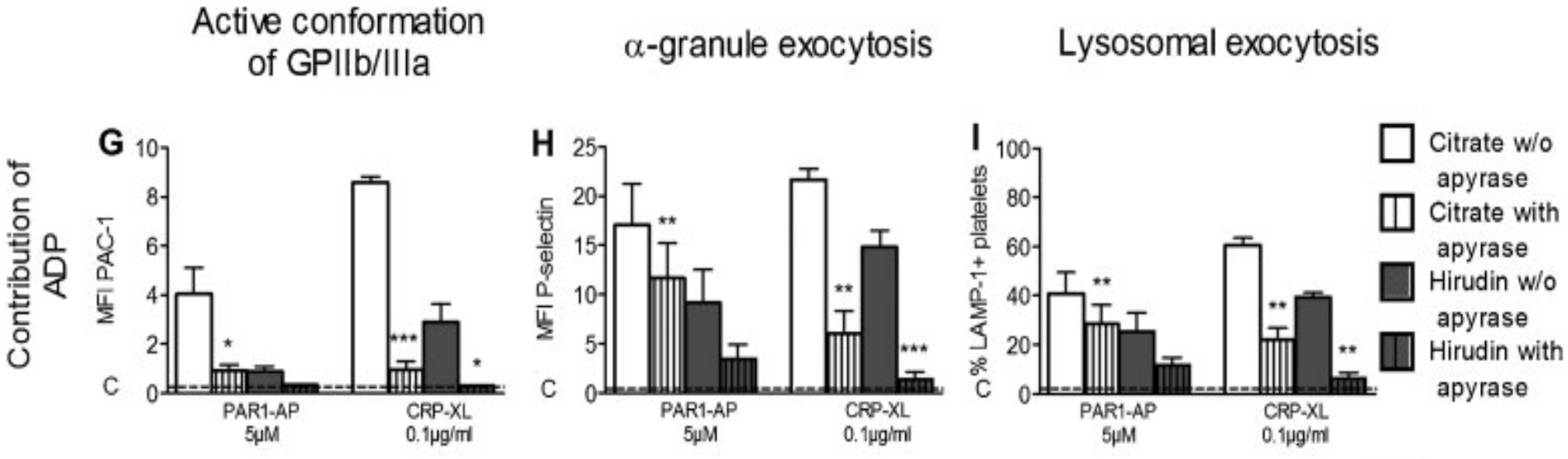
Impact of anticoagulants



Platelet responses are higher in citrated blood
(but still this is most commonly used)

Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

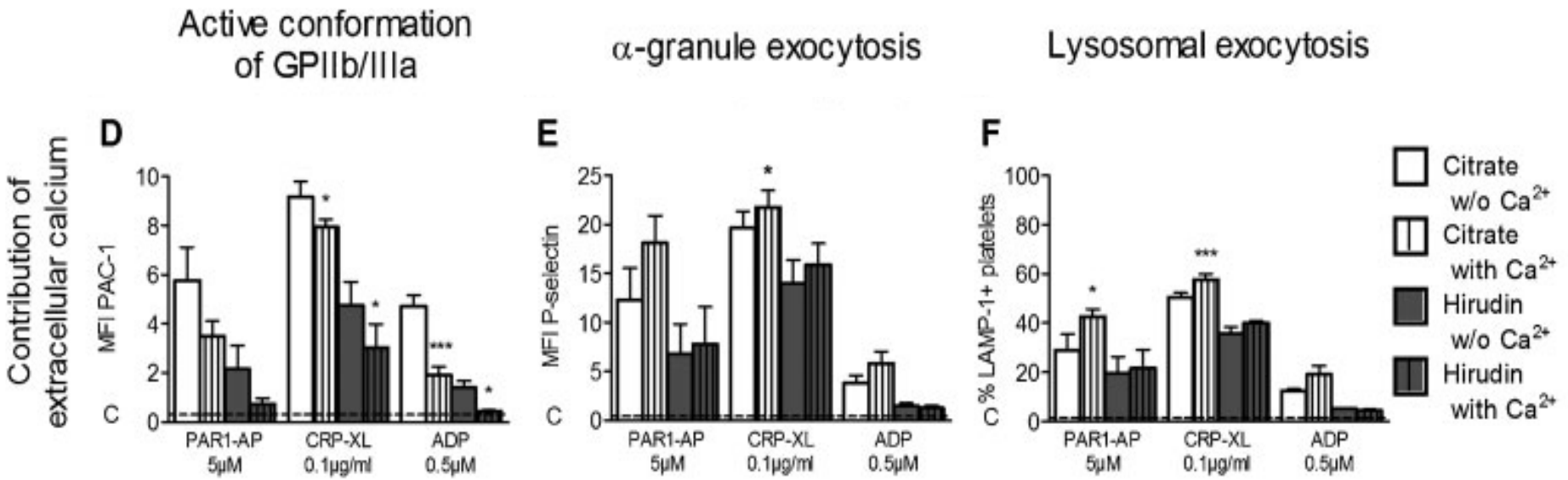
Platelet agonists – influence by autocrine ADP activation



Large influence by endogenous ADP, especially for fibrinogen receptor activation

Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

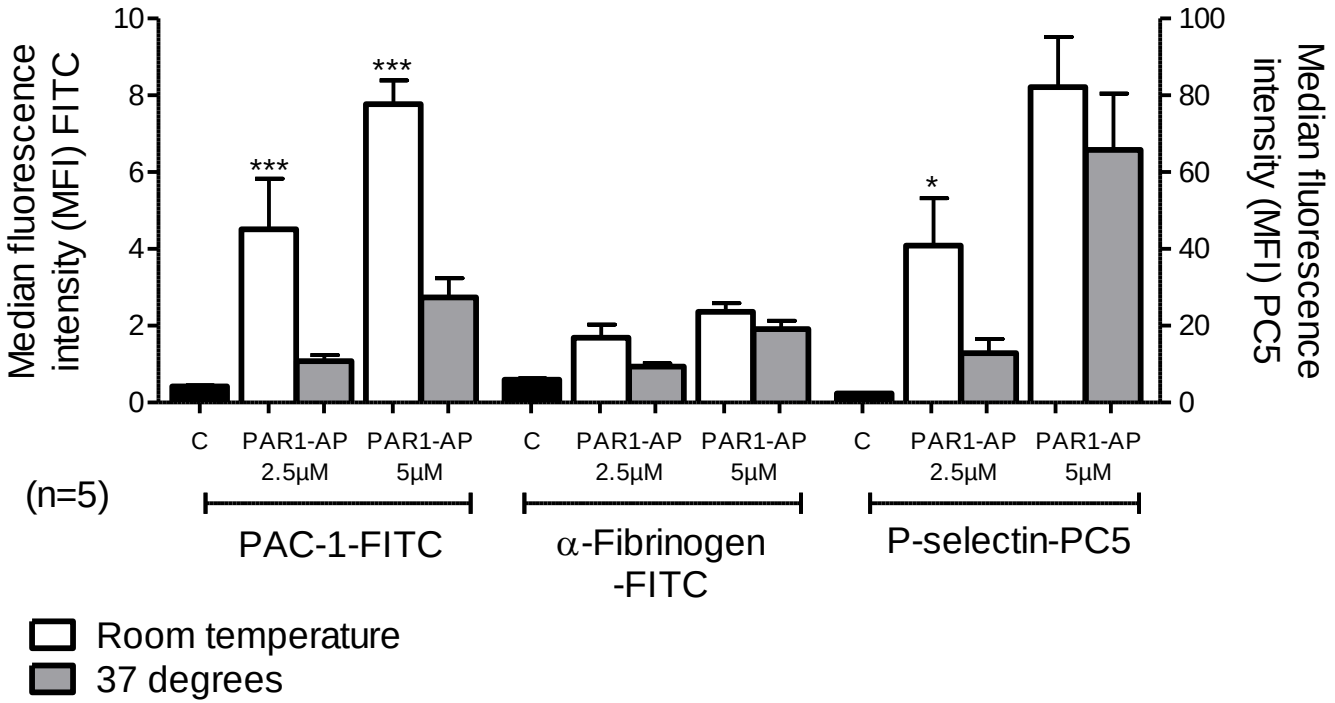
Impact of calcium in buffer



- Calcium in buffer reduces PAC-1 binding
- Calcium in buffer enhances granule secretion

Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

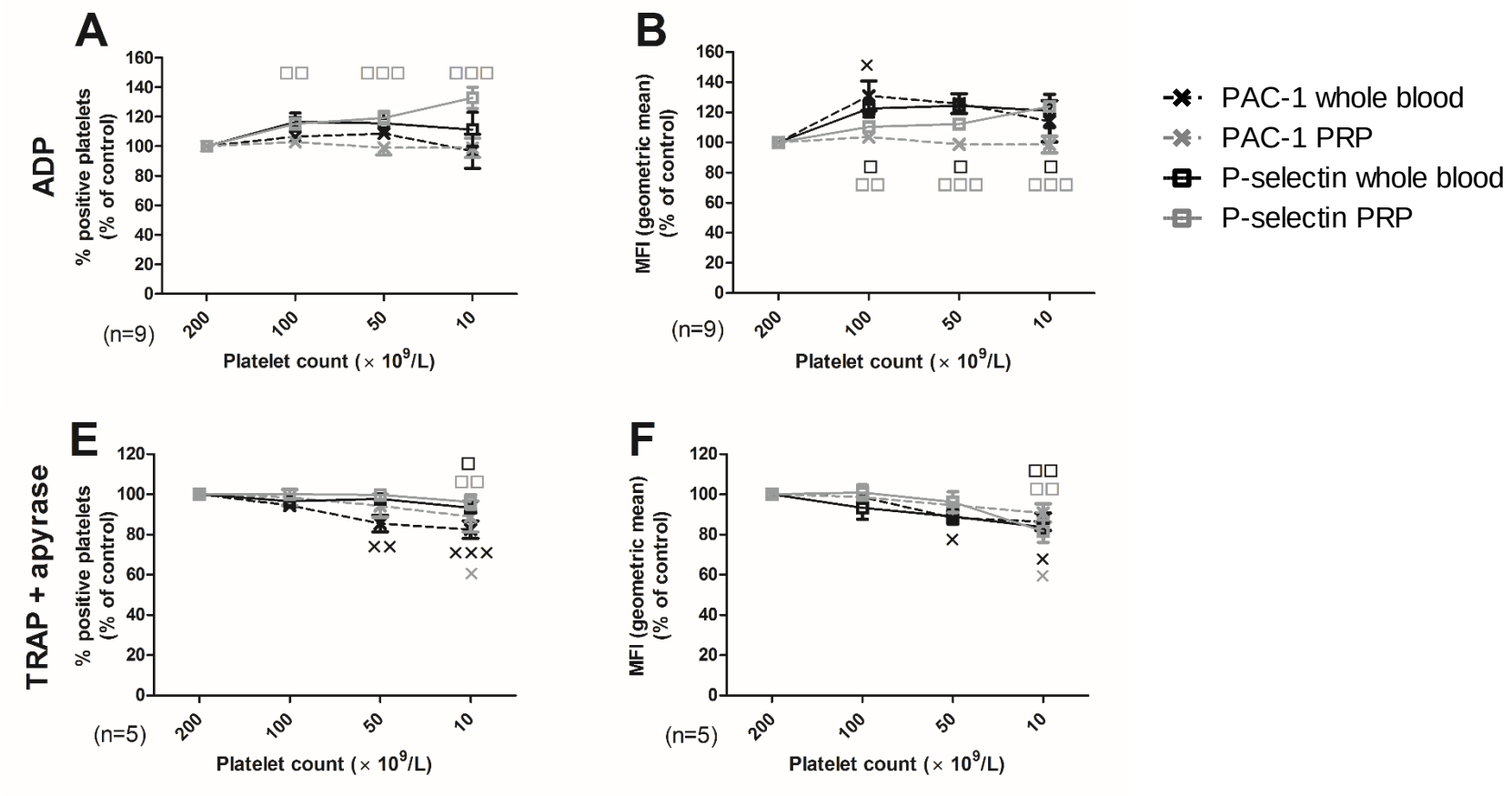
Impact of temperature



Platelet activation responses are higher at room temperature
(but this is still commonly used due to convenience and to avoid variability)

Ramström S, Södergren AL, Tynngård N, Lindahl TL. Platelet function determined by flow cytometry - new perspectives? *Semin Thromb Hemost.* 2016 Apr;42(3):268-81

Impact of sample platelet count

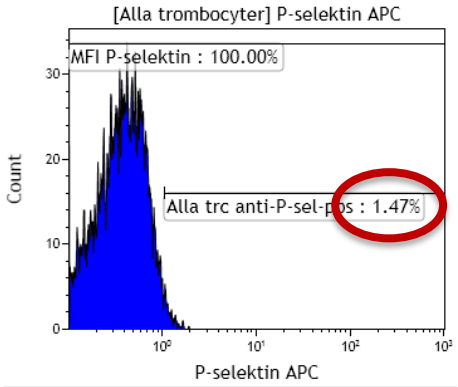


Platelet count affect autocrine activation, especially fibrinogen receptor activation (but much smaller effects than for aggregometry)

Boknäs N, Macwan AS, Södergren AL, Ramström S. Platelet function testing at low platelet counts: When can you trust your analysis? Res Pract Thromb Haemost. 2019 19;3(2):285-290.

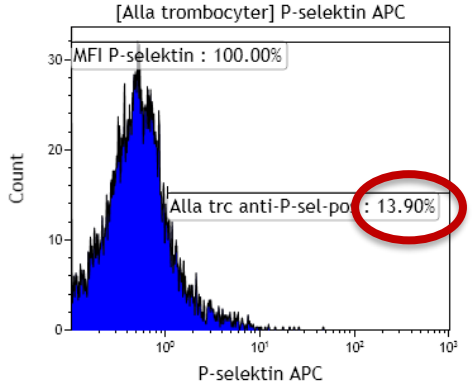
Special considerations – % positive platelets or MFI?

Fluorescence background (isotype control etc. Note: exact matching of F/P!)



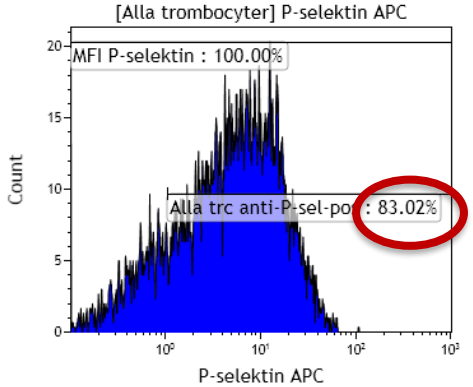
Gate	Number	%Gated	X-Med
All	5 170	100.00	0.34
Alla trc anti-P-sel-pos	76	1.47	1.19
MFI P-selektin	5 170	100.00	0.34

Spontaneous activation (buffer) – important to report!



Gate	Number	%Gated	X-Med
All	5 274	100.00	0.48
Alla trc anti-P-sel-pos	733	13.90	1.62
MFI P-selektin	5 274	100.00	0.48

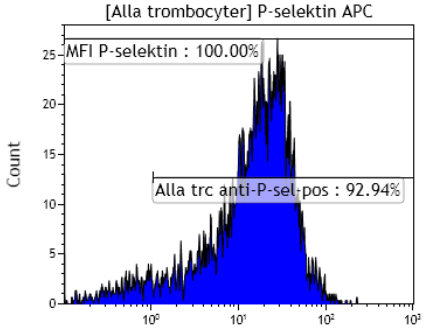
Medium strong agonist (less than 100 % positive)



Gate	Number	%Gated	X-Med
All	5 113	100.00	5.09
Alla trc anti-P-sel-pos	4 245	83.02	6.71
MFI P-selektin	5 113	100.00	5.09

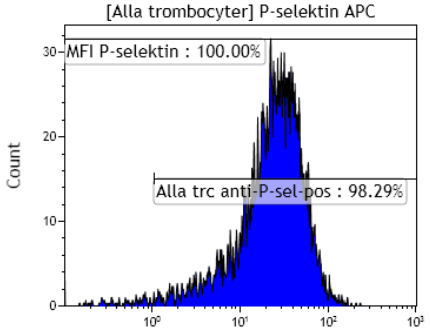
→ % positive platelets good readout

Strong agonist



Gate	Number	%Gated	X-Med
All	5 140	100.00	17.20
Alla trc anti-P-sel-pos	4 777	92.94	18.58
MFI P-selektin	5 140	100.00	17.20

Even stronger agonist



Gate	Number	%Gated	X-Med
All	5 088	100.00	25.21
Alla trc anti-P-sel-pos	5 001	98.29	25.61
MFI P-selektin	5 088	100.00	25.21

→ Median/geoMean fluorescence intensity good readout (but beads are needed for standardization between instruments)

Percent positive platelets – challenges with consistent gating

Thromb Haemost 1998; 79: 885–96

Review Article

European Working Group on Clinical Cell Analysis: Consensus Protocol for the Flow Cytometric Characterisation of Platelet Function

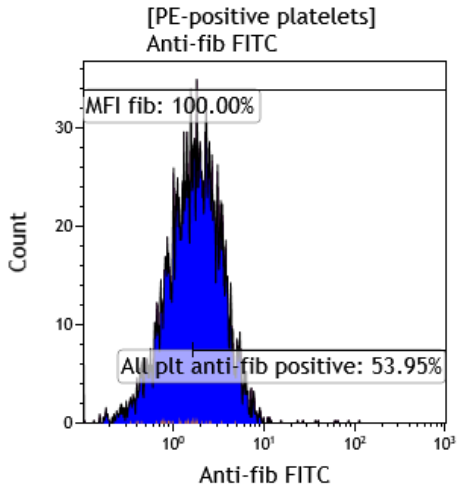
Gerd Schmitz, Gregor Rothe, Andreas Ruf[1], Stefan Barlage, Diethelm Tschöpe[2], Kenneth J. Clemetson[3], Alison H. Goodall[4], Alan D. Michelson[5], Alan T. Nurden[6], T. Vincent Shankey[7], for the European Working Group on Clinical Cell Analysis

Determination of "percent positive" Cells

This method is based on the quantitation of a small subpopulation of "positive" platelets expressing activation-associated antigens such as CD62P at low density in comparison to the "negative" fluorescence of a predominant platelet population. A threshold value for the "positive" signal intensity is defined based on an appropriate value for "false-positive" events, e.g., 1-2% in a 1-parameter histogram analysis of a matched control sample stained for the determination of non-specific fluorescence (see control samples). A threshold which reflects

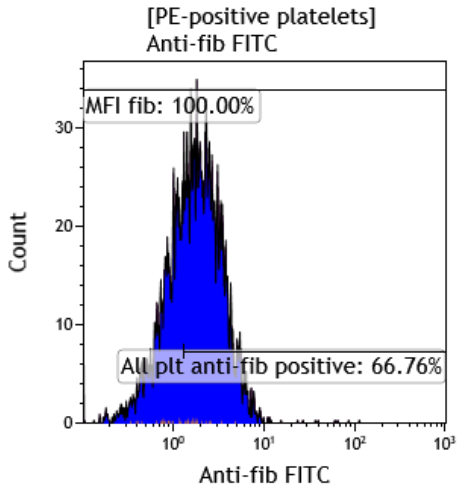
Percent positive platelets – challenges with consistent gating

Gate at 1%:



Gate	Number	%Gated
All	5 099	100.00
All plt anti-fib positive	2 751	53.95
MFI fib	5 099	100.00
Gate	**X-Med**	**X-CV**
All	1.74	130.44
All plt anti-fib positive	2.60	112.58
MFI fib	1.74	130.44
Gate	**HP X-CV**	
All	6.02	
All plt anti-fib positive	9.04	
MFI fib	6.02	

Gate at 2%:



Gate	Number	%Gated
All	5 099	100.00
All plt anti-fib positive	3 404	66.76
MFI fib	5 099	100.00
Gate	**X-Med**	**X-CV**
All	1.74	130.44
All plt anti-fib positive	2.32	115.33
MFI fib	1.74	130.44
Gate	**HP X-CV**	
All	6.02	
All plt anti-fib positive	6.02	
MFI fib	6.02	

→ For consistent gating we need to define a more specific threshold (we use 1.5%)

Anti-fibrinogen-FITC, platelets identified with anti-GPIb-PE, activation with ADP 5 µM, background fluorescence control sample: resting platelets, same ab but with 10 mM EDTA

Standardized gating - especially important in multi-center studies

Vox Sanguinis (2013) **105**, 38–46

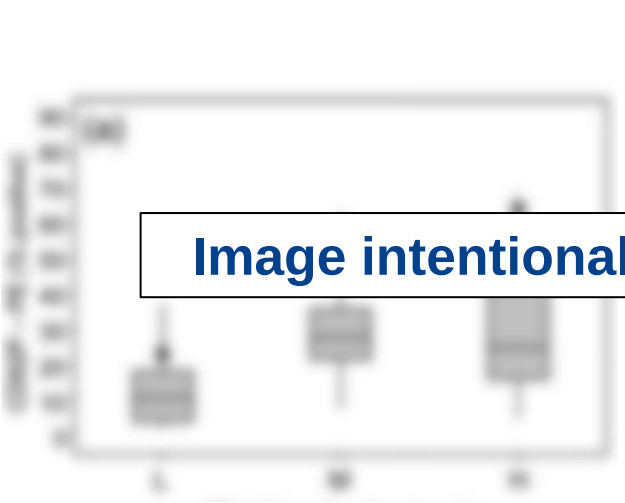
ORIGINAL PAPER

DOI: 10.1111/vox.12023

Standardization of CD62P measurement: results of an international comparative study

E. Levin, K. Serrano and D. V. Devine for the Biomedical Excellence for Safer Transfusion (BEST) Collaborative

Canadian Blood Services, University of British Columbia Centre for Blood Research, Vancouver, BC, Canada



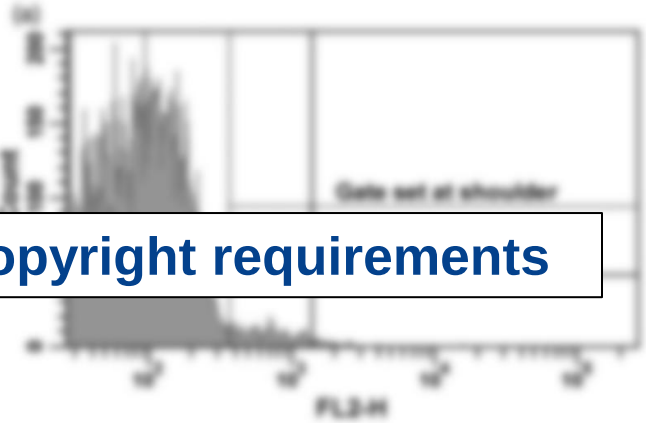


Image intentionally blurred to comply with copyright requirements

16 sites running the same fixed and pre-stained samples with low, medium and high P-selectin expression

Gating using 0.4% in isotype sample

Table 4 Intra- and interlaboratory CV (%) for CD62P

Percent positive platelets – challenges with gating

Platelets bind antibodies unspecifically

→ Important to account for this when analyzing low expressed activation markers

For activation markers: the background staining control should overlap with the resting platelet population!

→ Need to match both antibody type and F/P quote etc

FMO controls may be less suitable for platelets than for other cells?

MFI – advantages and challenges

+ No operator-dependent gating

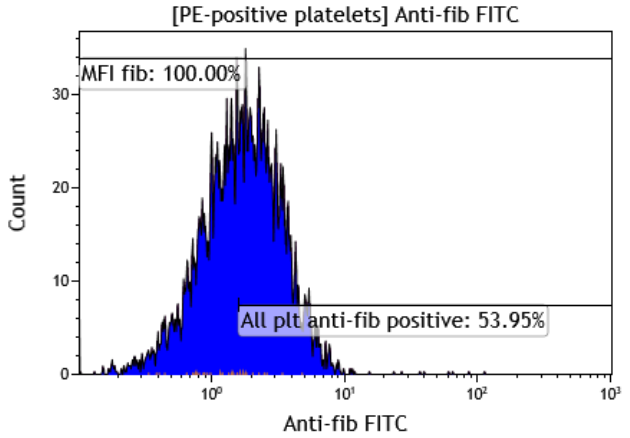
- Not sensitive for low expression

- Not useful if biphasic expression

- Data is not comparable between instruments

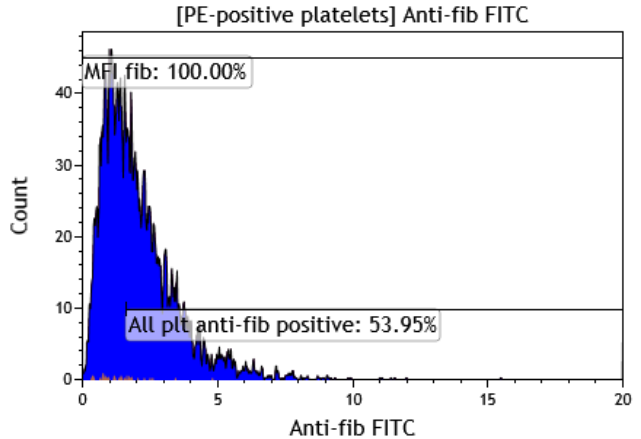
- Batch-to-batch variation for standardization beads

Logarithmic scale:



Gate	%Gated	X-Med	X-AMean	X-GMean
All	100.00	1.74	2.16	1.67
All plt anti-fib positive	53.95	2.60	3.15	2.78
MFI fib	100.00	1.74	2.16	1.67

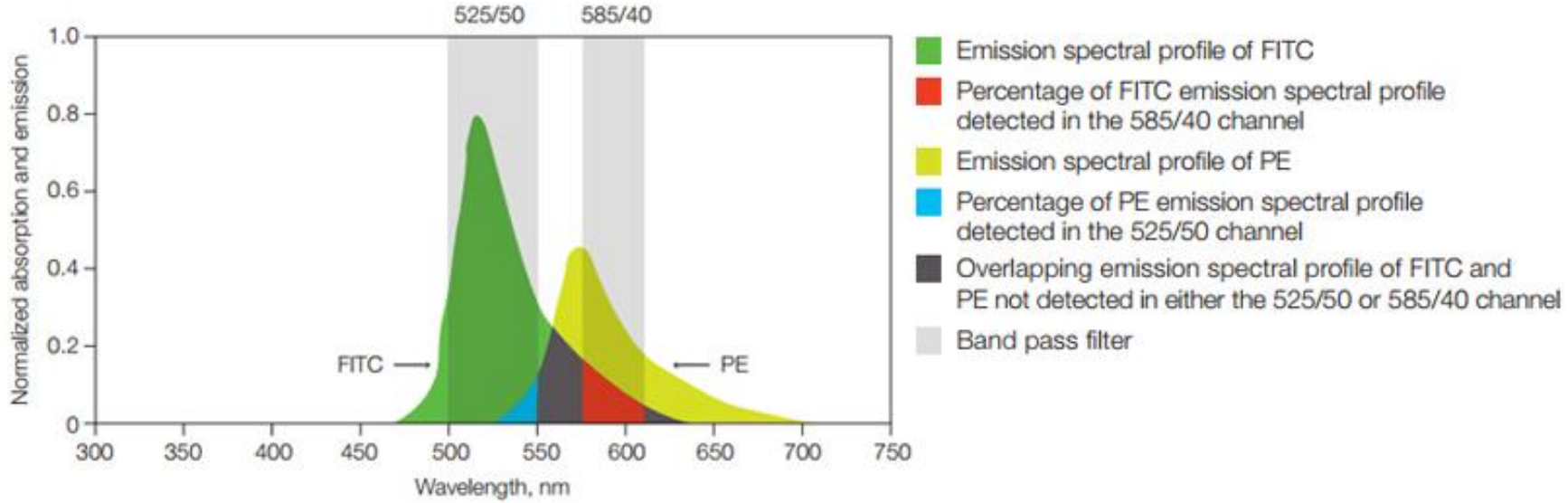
Linear scale:



Gate	%Gated	X-Med	X-AMean	X-GMean
All	100.00	1.77	2.16	0.93
All plt anti-fib positive	53.95	2.69	3.15	2.17
MFI fib	100.00	1.77	2.16	0.93

Median vs mean – remember that the expression is not normally distributed, it just appears to be in the logarithmic scale..

Compensation issues

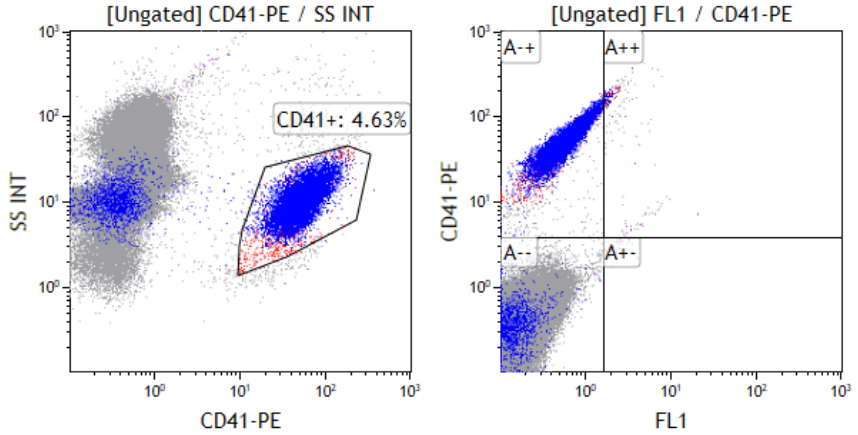


https://www.bio-rad-antibodies.com/flow-cytometry-fluorescence-compensation.html

- Important to consider and adjust
- Commoly seen percentages not always true for platelets
- Try to choose fluorophores with minimal overlap – or spectral flow cytometry

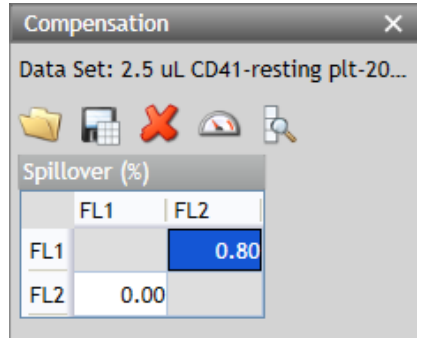
Compensation issues – need for adjustment if fluorescence for other markers changes with activation

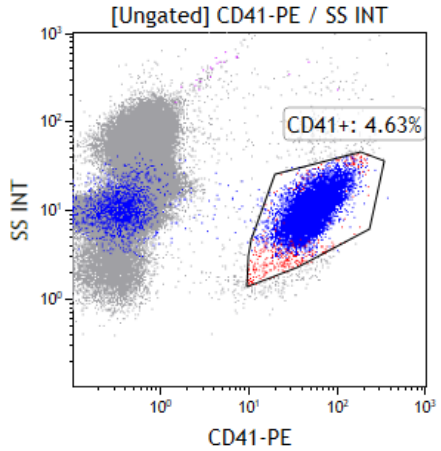
Resting sample, CD41-PE alone, no compensation:



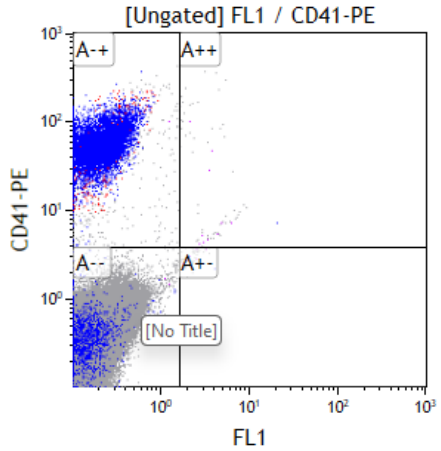
Gate	X-Med
All	0.49
CD41+	51.91

Gate	X-Med
All	0.19
A--	0.18
A-+	0.58
A+-	2.32
A++	2.08





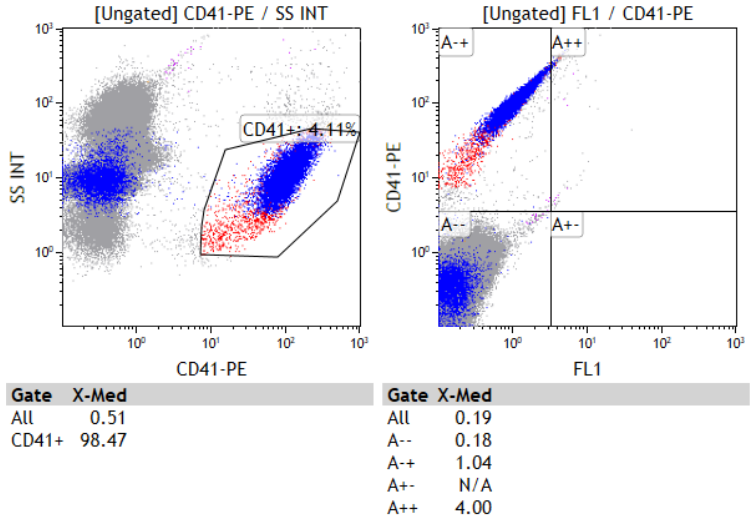
Gate	X-Med
All	0.49
CD41+	51.91



Gate	X-Med
All	0.17
A--	0.17
A-+	0.18
A+-	2.31
A++	3.51

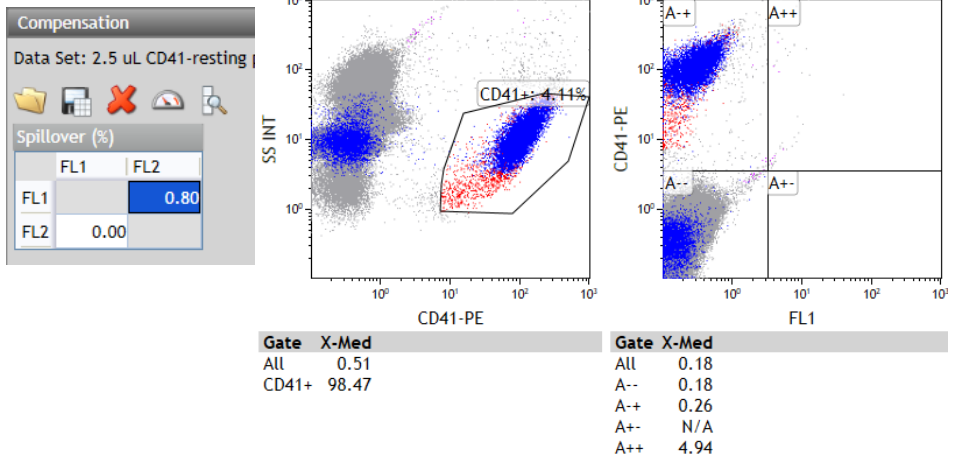
Compensation issues – need for adjustment if fluorescence for other markers changes with activation

Activated sample CD41-PE alone, no compensation:



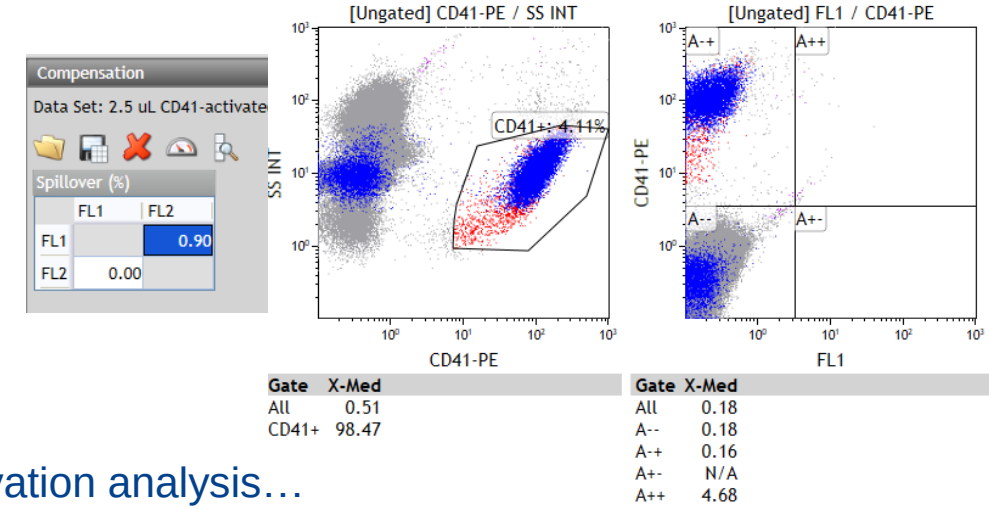
Gate	X-Med
All	0.51
CD41+	98.47

Gate	X-Med
All	0.19
A--	0.18
A-+	1.04
A+-	N/A
A++	4.00



Gate	X-Med
All	0.51
CD41+	98.47

Gate	X-Med
All	0.18
A--	0.18
A-+	0.26
A+-	N/A
A++	4.94



Gate	X-Med
All	0.51
CD41+	98.47

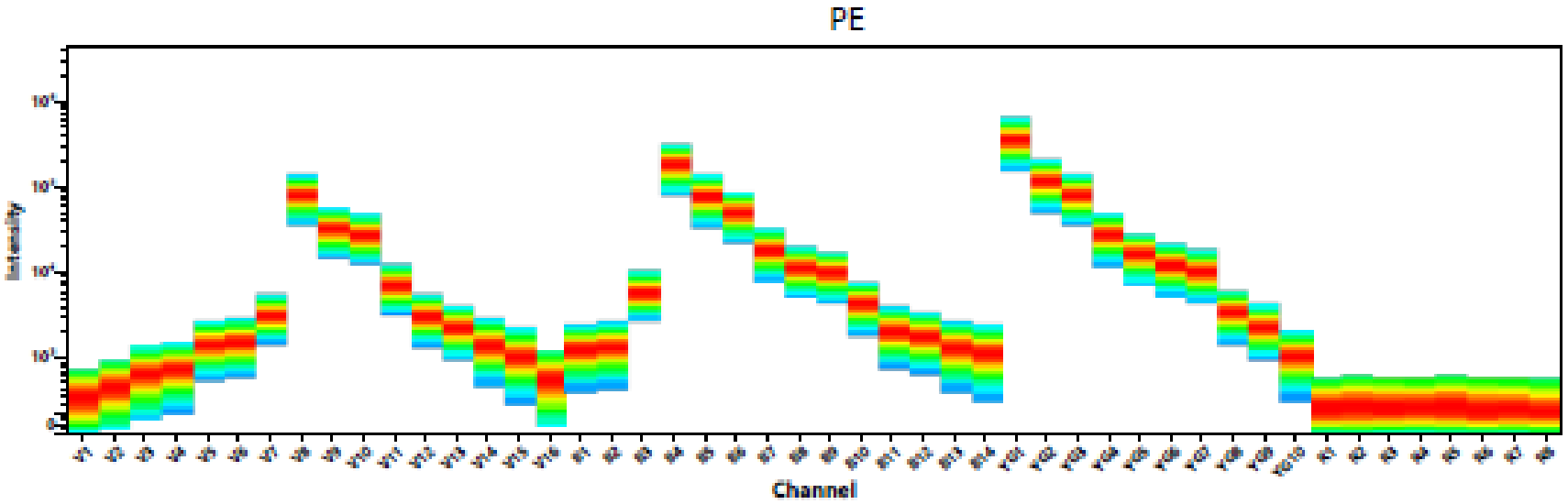
Gate	X-Med
All	0.18
A--	0.18
A-+	0.16
A+-	N/A
A++	4.68

And this is the case for most markers used for platelet activation analysis…
→ Avoid investigation of low expressed activation-induced antigens under these conditions

Multicolor protocols

If possible – always choose fluorophores with minimal compensation needs

Alternative option: spectral flow cytometry – avoids compensation by analysing the whole spectrum of each fluorophore



Multicolor protocols
– powerful but challenging to analyze and to interpret

Image intentionally blurred to comply with copyright requirements

Spurgeon, B. E. J., & Frelinger III, A. L. (2023). Platelet phenotyping by full spectrum flow cytometry. *Current Protocols*, *3,* e687. doi: 10.1002/cpz1.687

Challenges – platelet function testing in suspected PFD

- Large variation among healthy individuals
  → wide reference ranges
- Patients with well defined defects are very rare
  → cumbersome to validate protocols
- Many variables → hard to present in a pedagogical way
- Limited data correlating different variables to bleeding problems
- Interpretation even harder in cases with combinations of thrombocytopenia and PFD

Platelet function testing by flow cytometry - things to keep in mind

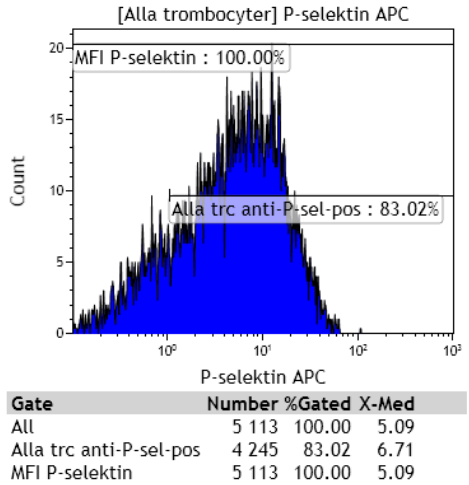
- We need to dilute samples and incubate under static conditions to avoid aggregation → less/no effect of TXA_2 autoactivation
- Presence of calcium is needed for some markers (e.g. Annexin V) and for normal functional responses – but need to prevent clotting if citrate was used as anticoagulant
- Citrate anticoagulation affect platelet activation potential – risk to misinterpret functional defects? (not just for FC)
- Still limited data available regarding the correlation between FC findings and bleeding score results
- Surface receptor analysis – quantitative defects will be detected but not qualitative (unless it affects the binding of the antibody used for detection)

Platelet function testing by flow cytometry - things to keep in mind

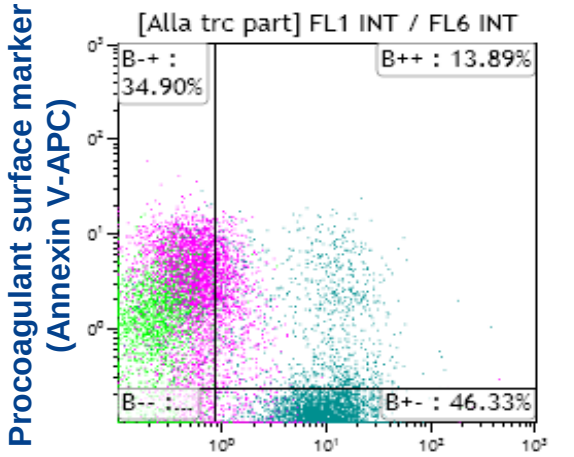
We will only study the circulating population of platelets (and with PRP perhaps not even all those)

Large variation in activation within the platelet population - and between healthy individuals

Platelet subpopulations? Important to study dot plots and not just numbers



Gate	Number	%Gated	X-Med
All	5 113	100.00	5.09
Alla trc anti-P-sel-pos	4 245	83.02	6.71
MFI P-selektin	5 113	100.00	5.09



platelets

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Check for updates

Variation in activation marker expression within the platelet population – a new parameter for evaluation of platelet flow cytometry data

Nahreen Tynngård[1,2], Aseel Alshamari[3,4], Freja Månsson[4], & Sofia Ramström[4,5]

Platelet function testing in suspected PFD – what is your aim?

- Evaluate the most important functions necessary for haemostasis?
- Detect/identify/investigate new defects in less known functions/receptors?

→ The answer to these questions should guide your choice of panel

Thank you for your attention!

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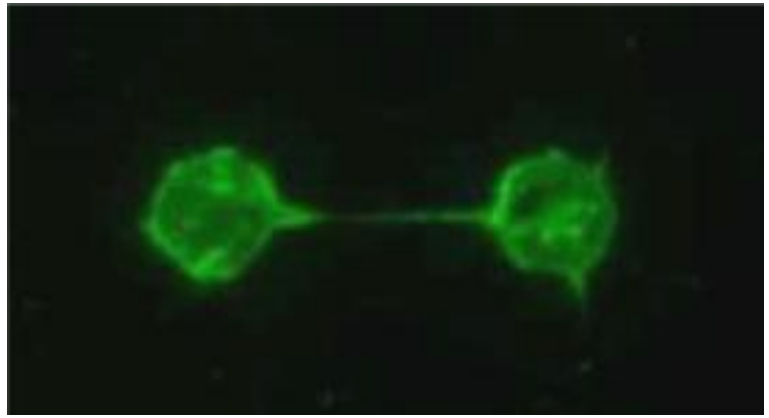
Want to get in contact?



sofia.ramstrom@oru.se

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https://x.com/crpp12

https://www.linkedin.com/company/fili%C3%A8re-de-sant%C3%A9-maladies-rares-mhemo/

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