Platelet Aggregation Assays

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International Society on Thrombosis and Haemostasis
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Disclosures for Tim Warner

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Honoraria	**No relevant conflicts of interest to declare**
Scientific Advisory Board	**No relevant conflicts of interest to declare**

Presentation includes discussion of the following off-label use of a drug or medical device:

<N/A>

Introduction

- Platelet aggregation assays
  - Diagnosis of bleeding disorders
  - Optimisation of anti-platelet therapy
  - GRAVITAS, TRILOGY, ARCTIC



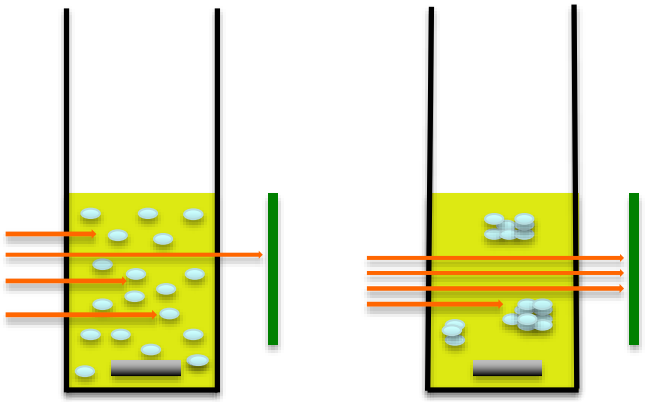






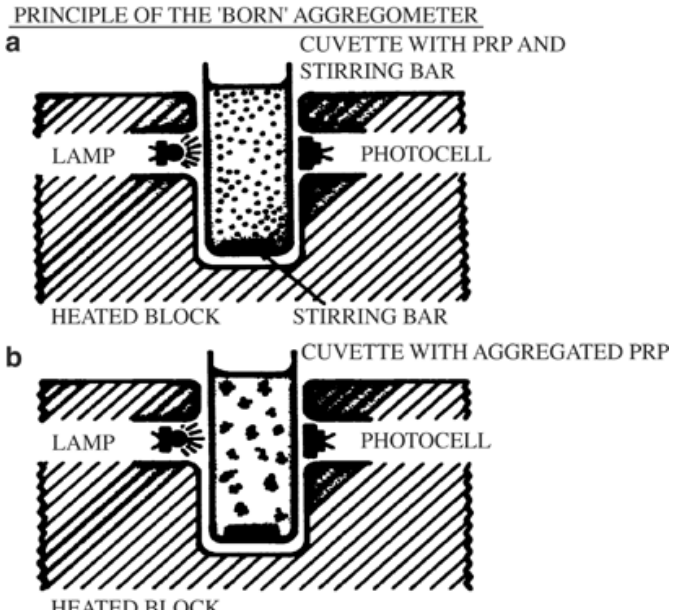
Introduction - PRP

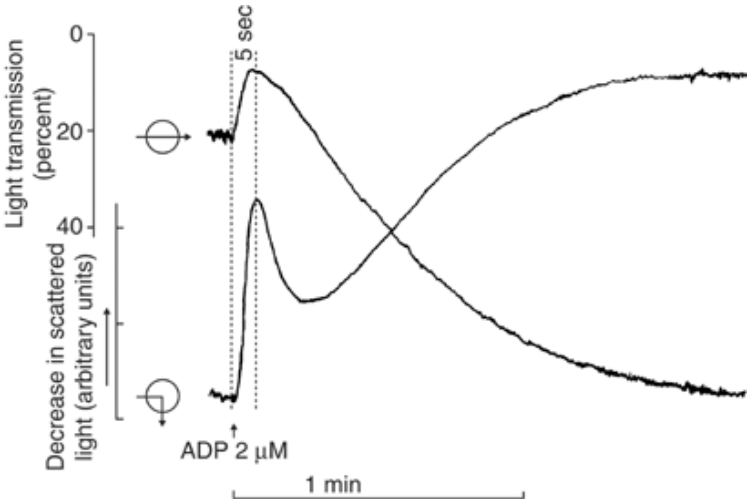
- Traditional light transmission aggregometry (LTA) is the ‘gold standard’
  - Experienced staff
  - Time-consuming
  - Labour-intensive
  - Relatively large volume of blood required
- LTA methodologies and outputs vary widely between laboratories
  - Blood collection, storage, platelet-rich plasma (PRP) preparation
  - LTA technique
    - Agonists & concentrations
    - Incubation times
    - Operator differences
  - Data acquisition and calculation



Measuring platelet aggregation in platelet rich plasma (PRP)

- Light Transmission Aggregometry – Born, 1962

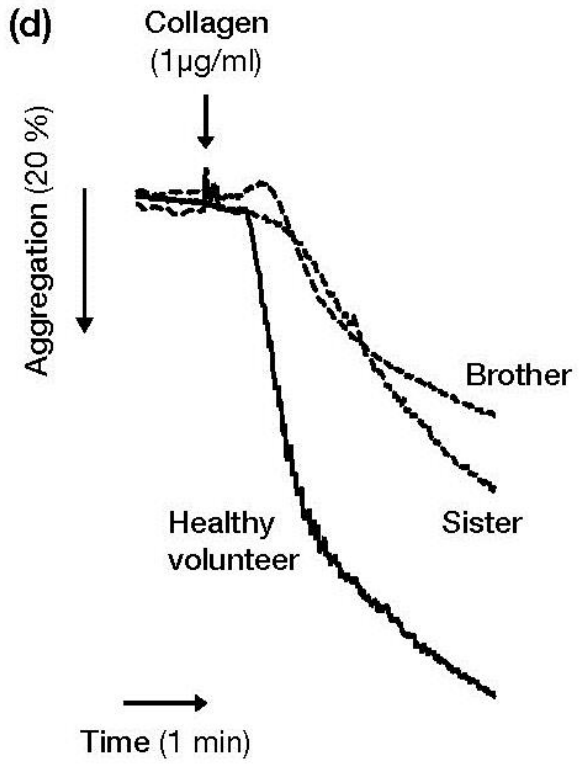






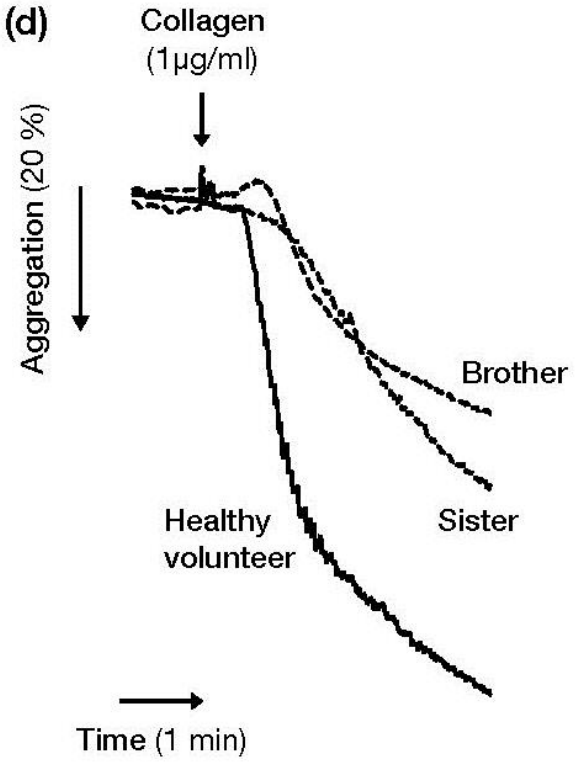
Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962 Jun 9;194:927-9.

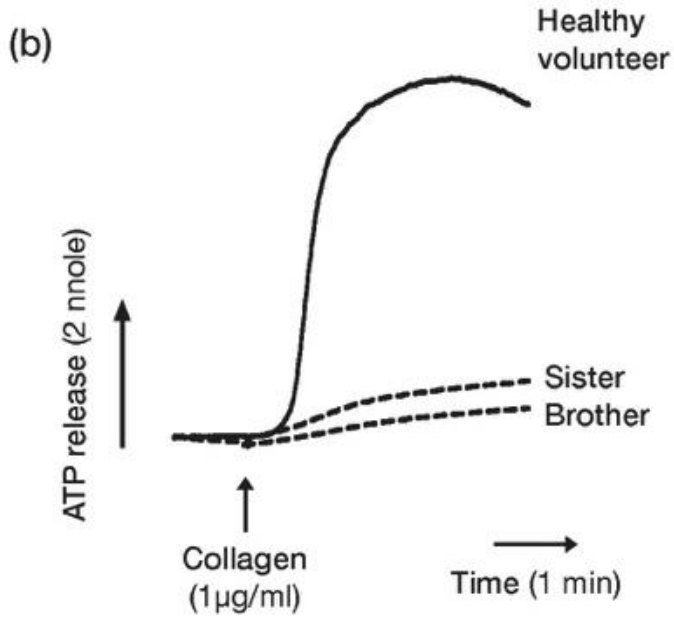
Example: using traditional LTA to measure platelet aggregation and ATP release in platelet rich plasma (lumi-aggregometry) to detect genetic disorder (dysfunctional $cPLA_{2\alpha}$)



Kirkby et al. FASEB J. 2015 Nov;29(11):4568-78.

Example: using traditional LTA to measure platelet aggregation and ATP release in platelet rich plasma (lumi-aggregometry) to detect genetic disorder (dysfunctional $cPLA_{2\alpha}$)



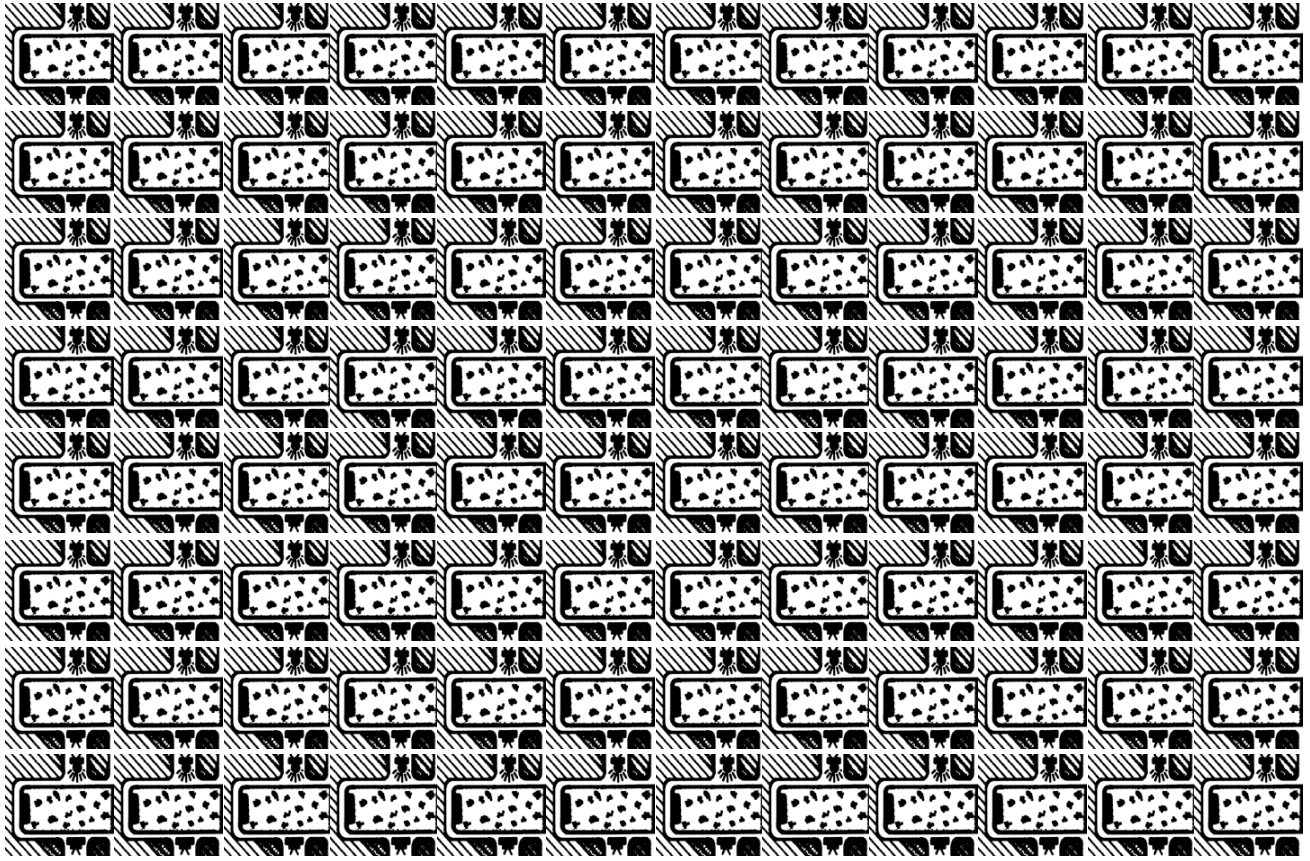


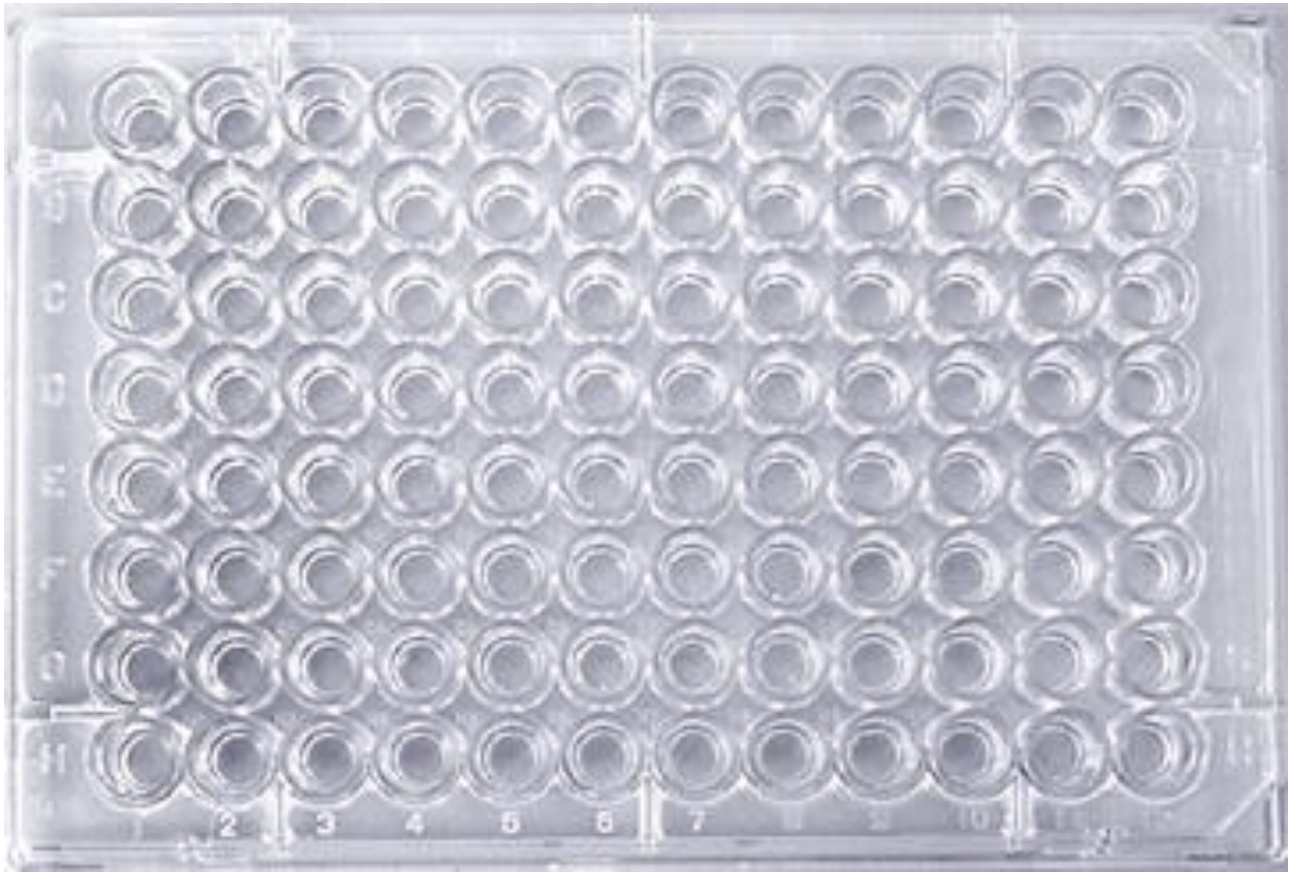
luciferase + ATP = light

Kirkby et al. FASEB J. 2015 Nov;29(11):4568-78.

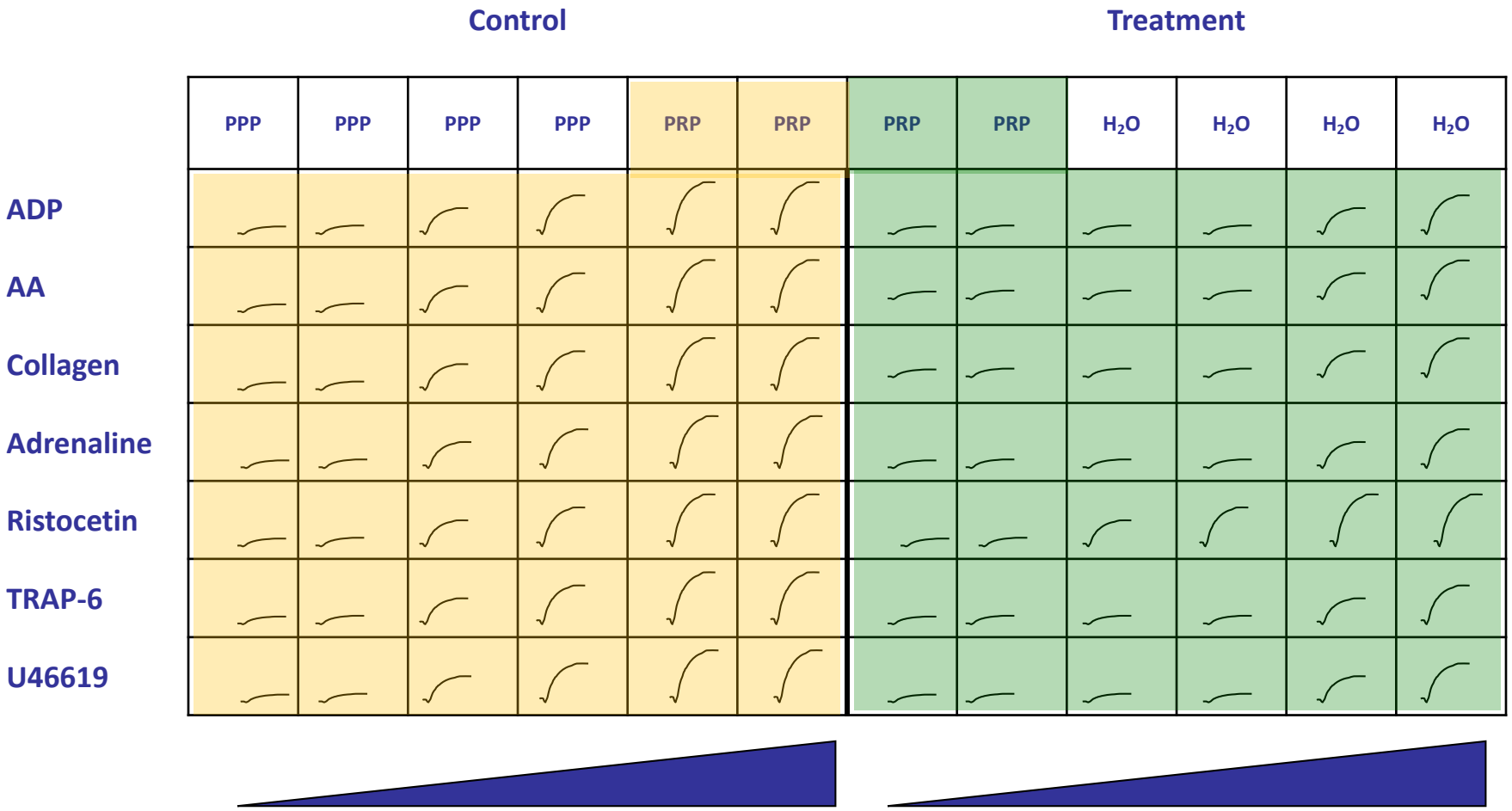




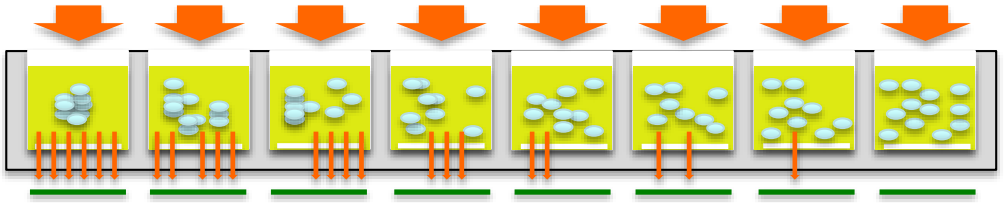



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Methods – example of plate set up for *in vitro* drug testing



96-well plate-based aggregometry



- Aggregation in wells read at the same time
  - Hours > Minutes
- Smaller volume of blood required than for LTA

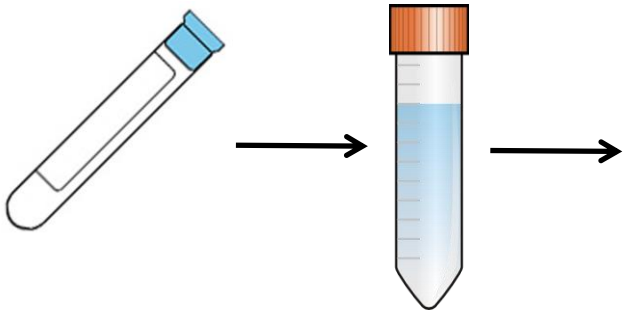
- Agonists still need to be freshly made, and mixing may be a variable

➢ Lyophilised 96-well half-area plates
  ➢ Only requires addition of PPP or PRP to appropriate wells

Methods: Optimul Plates

Whole blood

PRP



	1	2	3	4	5	6	7	8	9	10	11	12
A	PPP	AA 1 mM	ADP 40 µM	C 40 µg/ml	E 10 µM	T6 40 µM	U4 40 µM					
B	PPP	AA 0.57 mM	ADP 8.89 µM	C 10 µg/ml	E 1.82 µM	T6 12.3 µM	U4 8.89 µM					
C	PPP	AA 0.33 mM	ADP 1.98 µM	C 2.50 µg/ml	E 0.33 µM	T6 3.79 µM	U4 1.98 µM					
D	PPP	AA 0.19 mM	ADP 0.44 µM	C 0.62 µg/ml	E 0.06 µM	T6 1.17 µM	U4 0.44 µM					
E	PRP	AA 0.11 mM	ADP 0.10 µM	C 0.16 µg/ml	E 0.01 µM	T6 0.36 µM	U4 0.10 µM					
F	PRP	AA 0.06 mM	ADP 0.02 µM	C 0.04 µg/ml	E 0.001 µM	T6 0.11 µM	U4 0.02 µM					
G	PRP	AA 0.03 mM	ADP 0.005 µM	C 0.01 µg/ml	E 0.0004 µM	T6 0.03 µM	U4 0.005 µM					
H	PRP	AA Buffer	ADP Buffer	C Buffer	E Buffer	T6 Buffer	U4 Buffer					





5 minutes, 37° C

Chan et al. Platelets. 2011;22(7):485-94 / Chan & Warner. Platelets. 2012;23(5):404-8.

Methods: Platelet Aggregation

- Light transmission determined by a standard 96-well plate reader
- % aggregation was calculated by reference to the absorbances of PRP (0%) and PPP (100%)

- Platelet releasates
  - ATP/ADP - by luminescence
  - Thromboxane (TX) A_2 - by ELISA, for its stable metabolite TxB_2

- In some experiments, PRP was pre-incubated with aspirin (30µM), or $P2Y_{12}$ receptor antagonist, prasugrel active metabolite (PAM, 3µM).

Optimul plate reactivity is maintained at 12 weeks

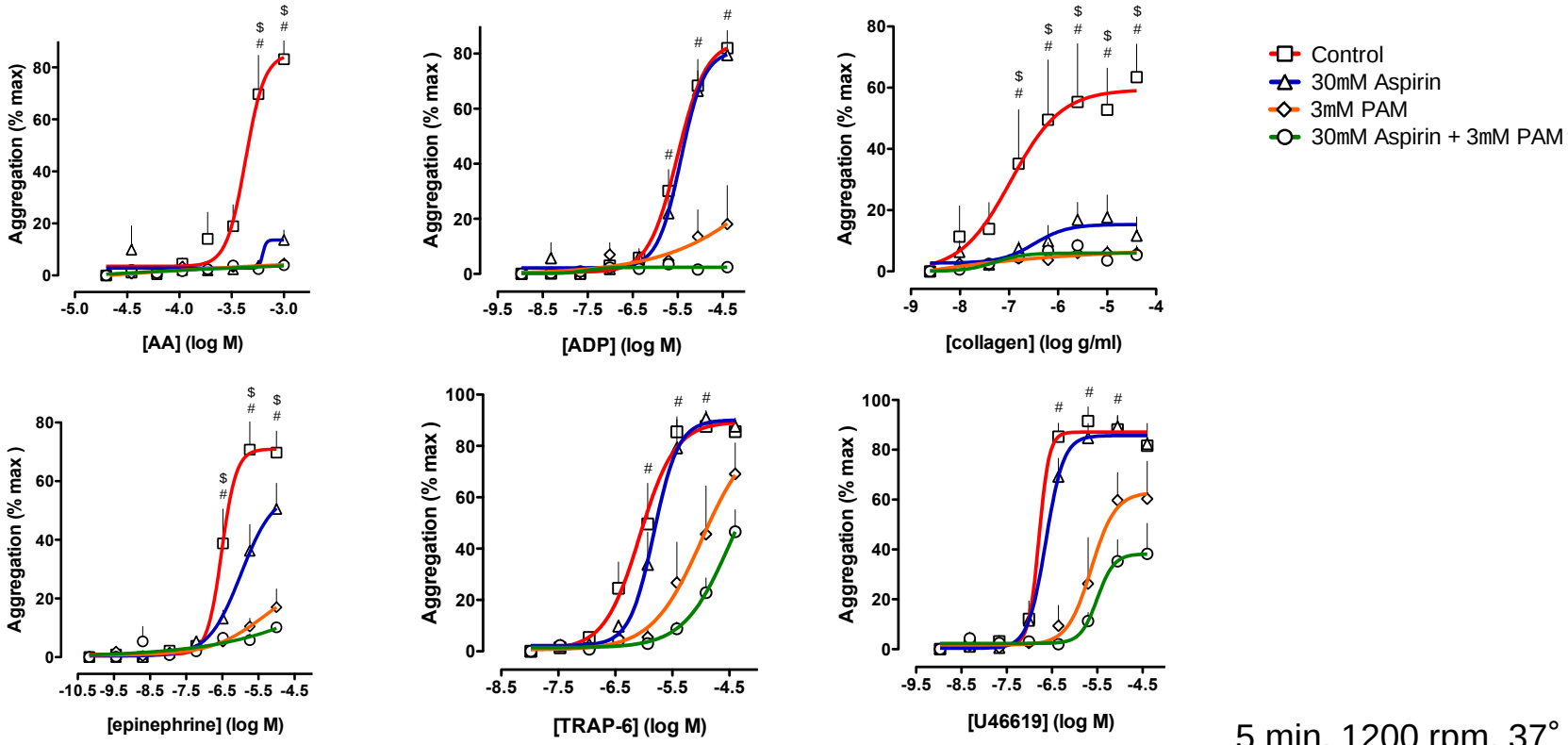
Maximal U-46619-induced aggregation

	Maximal aggregation (%)			
	Week 0	Week 3	Week 6	Week 12
AA	68±14	63±10	69±13	57±15
ADP	62±4	57±4	59±3	57±9
collagen	73±13	69±7	66±12	67±9
EPI	50±10	52±6	59±6	56±3
TRAP-6	66±5	60±4	70±4	69±8
U46619	79±4	71±1	77±3	84±4

Chan et al. Platelets. 2011;22(7):485-94 / Chan & Warner. Platelets. 2012;23(5):404-8.

Optimul PAM/ASA

- Aspirin caused inhibition of platelet aggregation responses to AA, EPI and collagen
- PAM inhibited platelet responses to AA, ADP, collagen, EPI, TRAP-6 and U46619



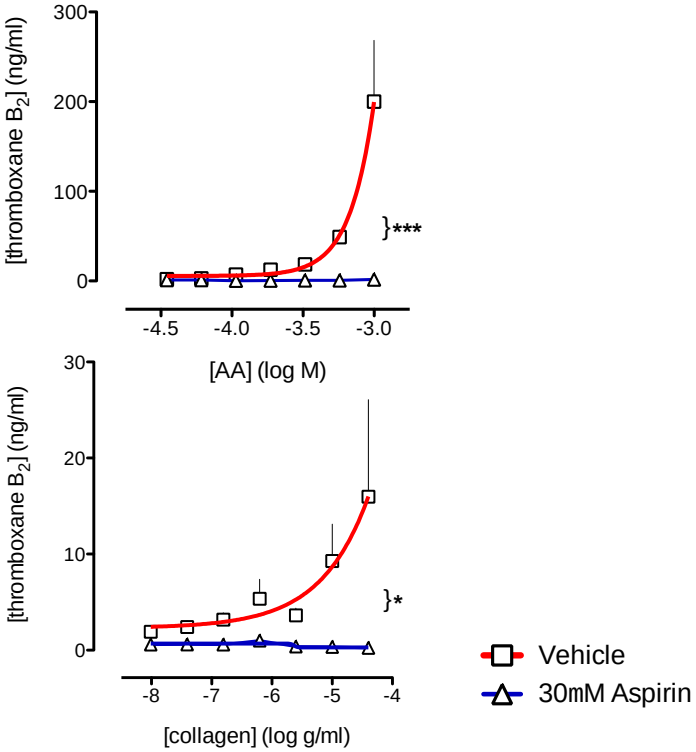
5 min, 1200 rpm, 37° C

Curves fitted by four-parameter logistic non-linear regression and data compared by paired two-way ANOVA with Bonferroni's post-tests indicated as $p<0.05$ with comparisons $ control vs. aspirin, # as control vs. PAM and * as PAM vs. aspirin. Data shown as mean±s.e.m. n = 6

Chan et al. Platelets. 2011;22(7):485-94 / Chan & Warner. Platelets. 2012;23(5):404-8.

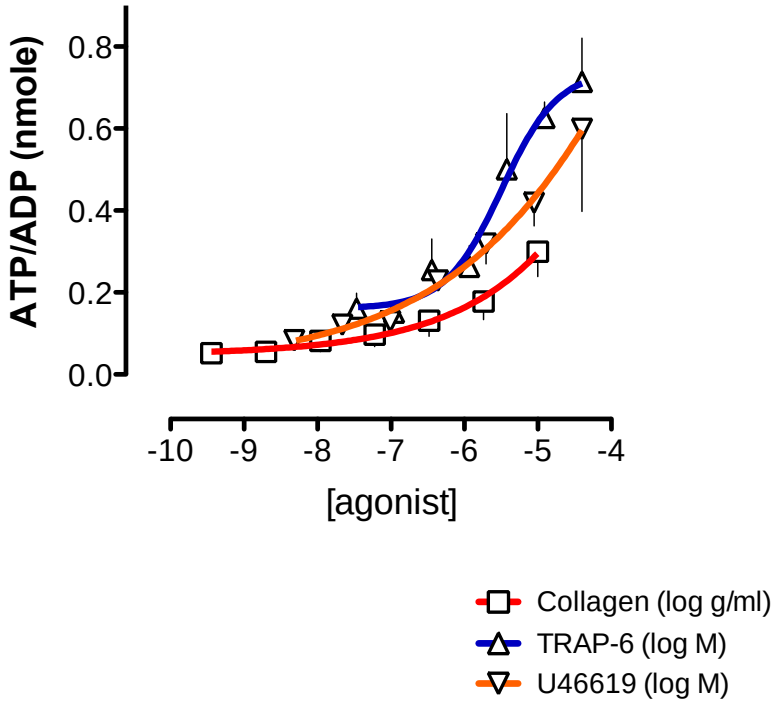
Detection of Platelet Releasates

- AA & collagen-induced TXA_2 production is abolished by aspirin



Platelet production of TXA_2, induced by AA (0.02-1 mM) or Collagen (0.002-40 µg mL^{-1}) in the presence of aspirin (30 µM) or vehicle, measured by ELISA. Curves fitted by four-parameter logistic non-linear regression and data compared by paired two-way ANOVA with $p<0.05$ shown as * and $p<0.001$ as ***. Data shown are mean ± s.e.m of responses measured in PRP prepared from n = 4.

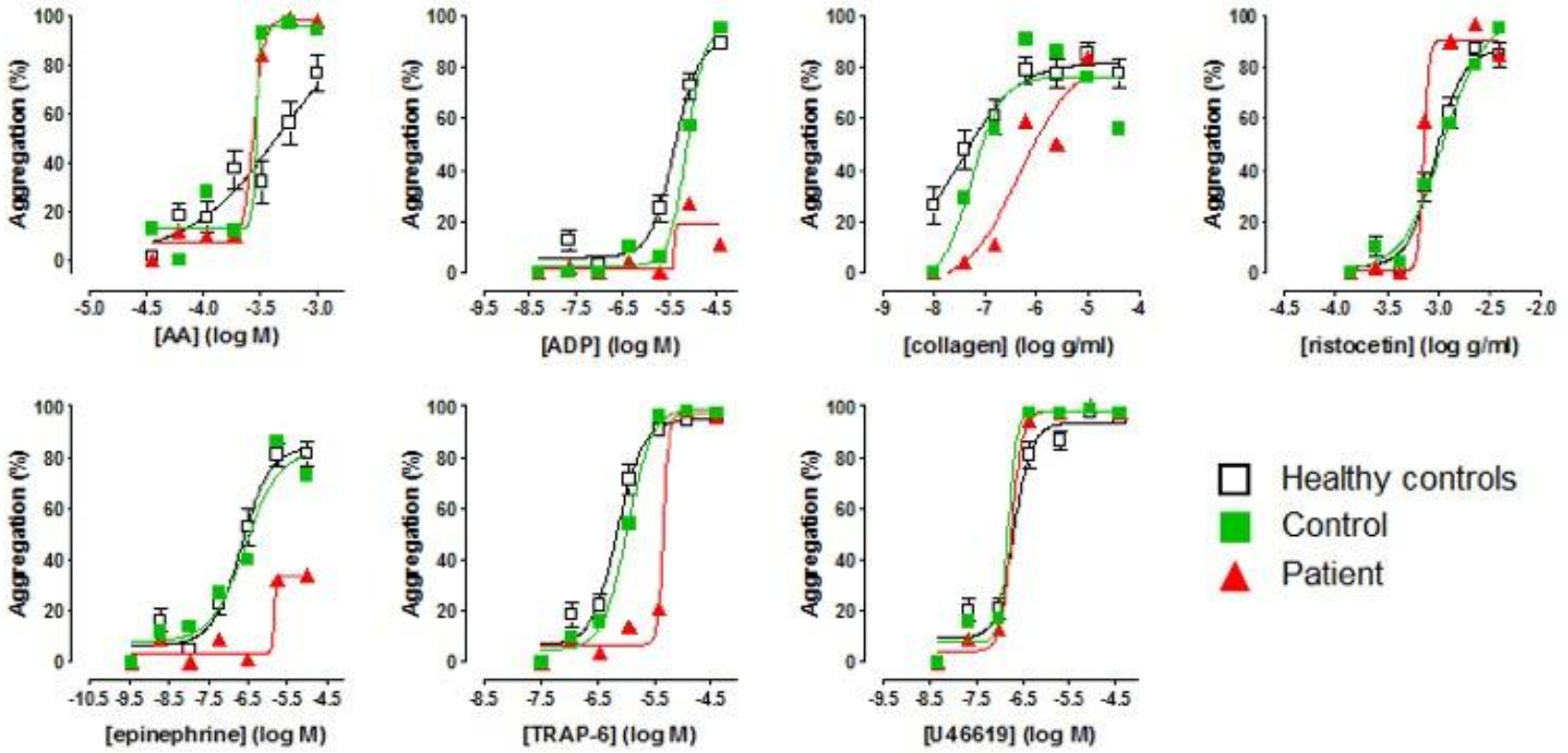
- Concentration-dependent release of ATP/ADP with collagen, TRAP-6 & U46619



The release of ATP+ADP from platelets stimulated with collagen (0.002-40 µg/mL), TRAP-6 (0.01-40 µM) or U-46619 (40 µM). Data shown are mean ± s.e.m of responses measured in PRP prepared from n = 7

Chan et al. Platelets. 2011;22(7):485-94 / Chan & Warner. Platelets. 2012;23(5):404-8.

LTA vs Optimul in bleeding disorder



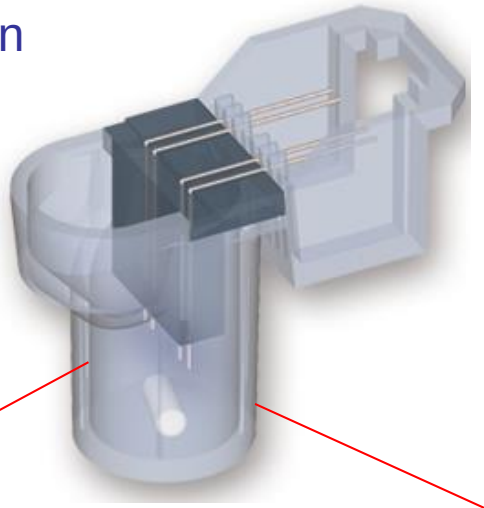
Lordkipanidzé et al. Blood. 2014 Feb 20;123(8):e11-22.

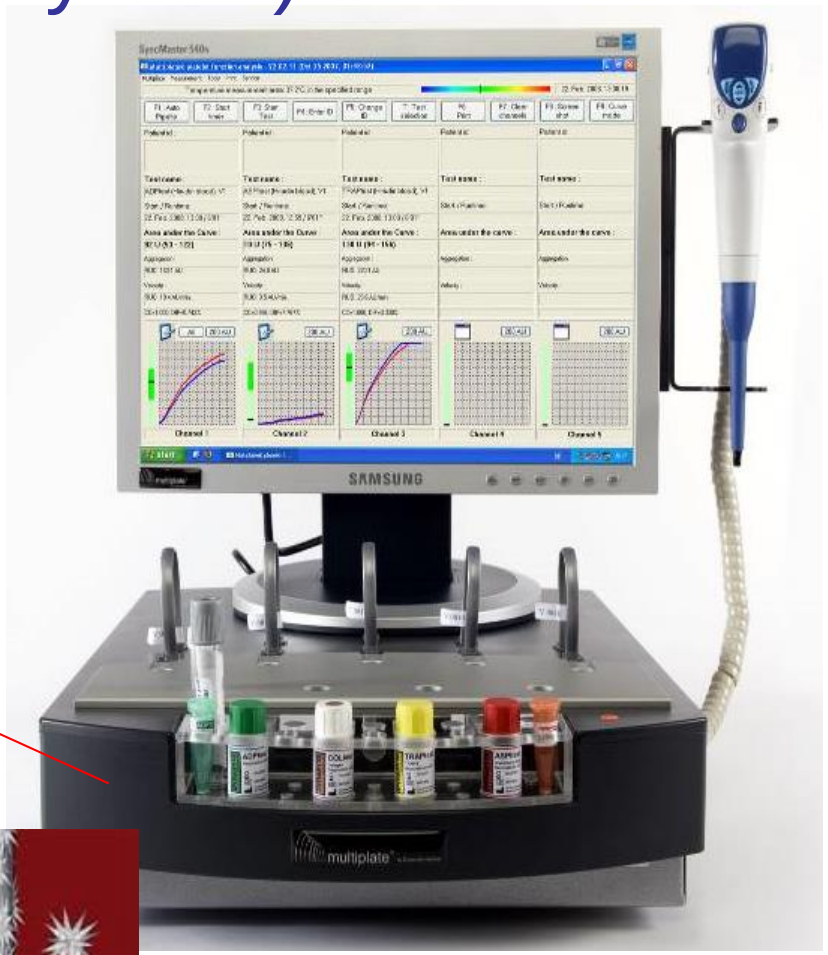
Optimul Summary

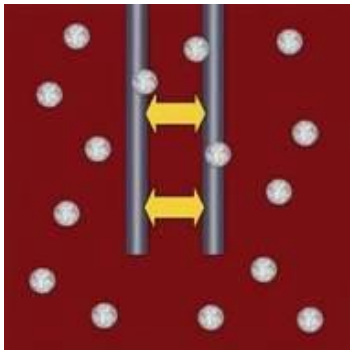
	LTA	optimul
Test type	Platelet aggregation TXA_2 release ATP/ADP release	Platelet aggregation TXA_2 release ATP/ADP release
Agonists types & concentrations used	Variable between sites	Centralised production leads to standardised agonists and concentrations
Agonist storage	All agonists must be used immediately	Plates may be stored for at least 3 months
Operator variability	Aggregometer brands and protocols vary	Simple endpoint assay with BioShake - Just add PPP and PRP
Data acquisition & calculation	Variable between operator/site	Standardised data collection and analysis through fixed statistical and graphing protocols
Time	Hours for thorough platelet analysis	Only 5 minutes from preparing PPP and PRP
Volume of blood required	Large for full platelet function analysis	Less than 2 ml PRP to construct full concentration-responses curves to 6 of the most relevant platelet agonists e.g. neonates
Use?		Inexperienced laboratories Remote testing Large cohort testing

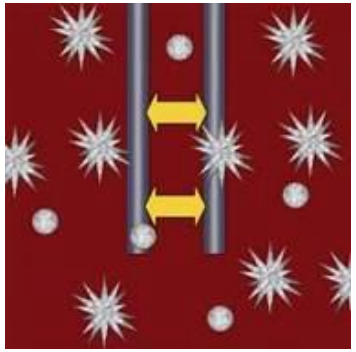
Whole blood impedance aggregometry (Multiplate® system)

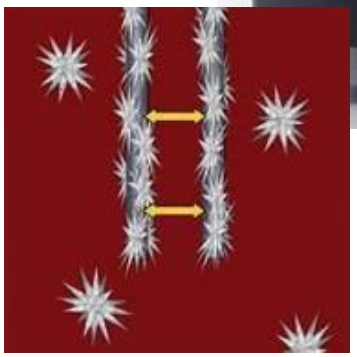
platelet function analysis in whole blood based on impedance aggregometry







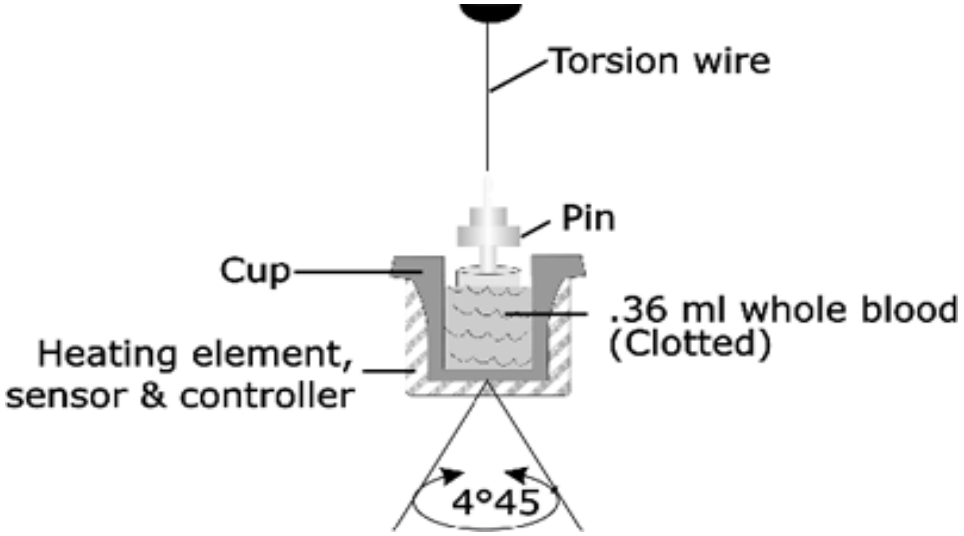




ROTEM & Haemoscope TEG®



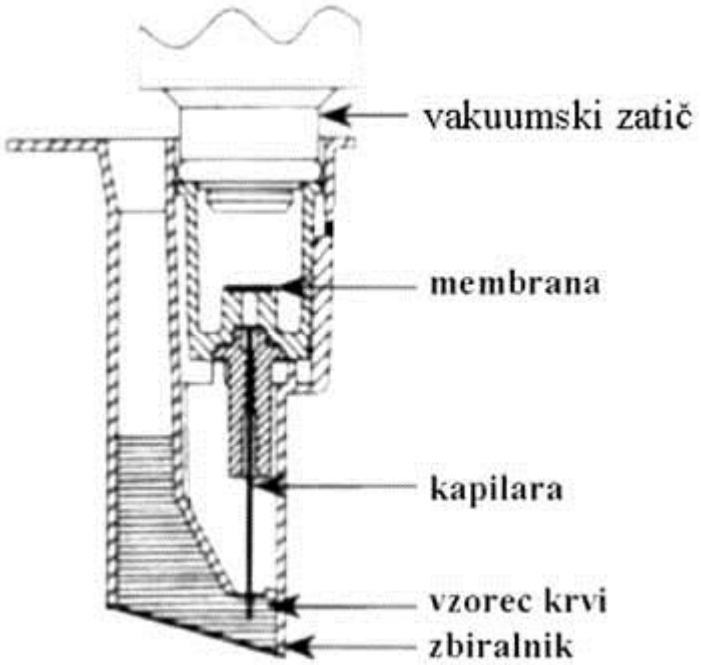




PFA-100®, high shear whole blood analyser

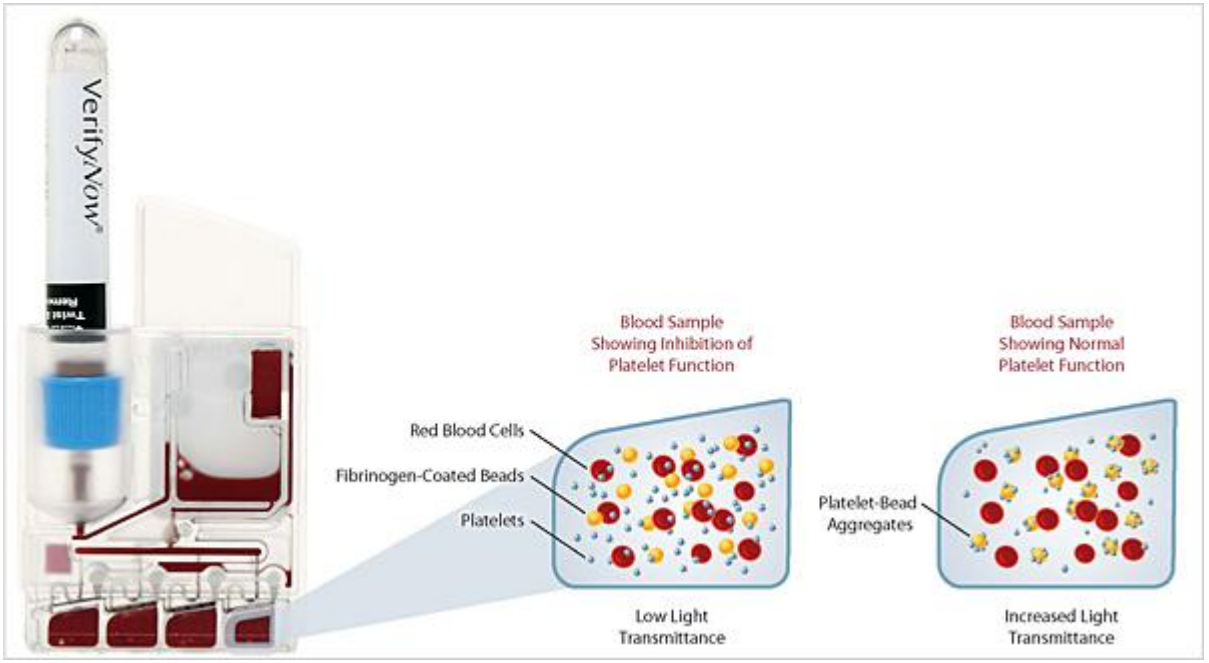






VerifyNow® whole blood analyser





Whole Blood Platelet Reactivity Assay

Thromb Haemost 2001; 86: 668–71
© 2001 Schattauer GmbH, Stuttgart

Serial Determinations of Platelet Counts in Mice by Flow Cytometry

Kishore R. Alugupalli[1], Alan D. Michelson[2], Marc R. Barnard[2], John M. Leong[1]

[1]Department of Molecular Genetics and Microbiology, [2]Center for Platelet Function Studies, University of Massachusetts Medical School, Worcester, Massachusetts, USA

Platelets. 2004 Mar;15(2):85-93.

Quantitation of platelet aggregation and microaggregate formation in whole blood by flow cytometry.

Fox SC, Sasae R, Janson S, May JA, Heptinstall S.

Cardiovascular Medicine, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH, UK. sue.fox@nottingham.ac.uk

Abstract

Platelet aggregation and microaggregate formation were measured in samples of stirred whole blood by flow cytometry. Blood samples were stirred in a multi-sample agitator with ADP, fixed and labelled with a platelet-specific CD42a-FITC fluorescent antibody. The blood was then diluted and applied directly to a flow cytometer. Platelets were identified using a gating procedure based on their expression of CD42a and then quantified. Aggregation was monitored as a fall in the number of single platelets. Both reversible and irreversible aggregation responses to ADP were determined and these were found to correlate directly with aggregation responses determined using a well-established single platelet counting technique using the Ultra-Flo 100 Whole Blood Platelet Counter. We found from flow cytometry that ADP-induced aggregation was coupled with a transient formation of platelet microaggregates over the initial 60 s following ADP addition. Assessment of single platelet loss by flow cytometry was found to be a reliable way of monitoring aggregation responses and provided new information on rapid microaggregate formation in ADP-stimulated blood.

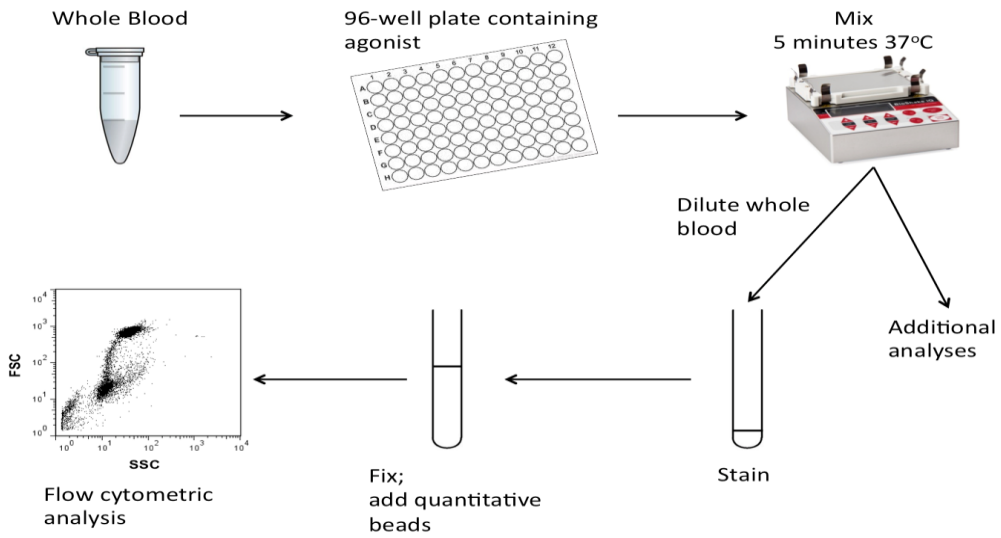
PMID: 15154600 [PubMed - indexed for MEDLINE]

Benefits

- Less blood volume
- High-throughput

Whole Blood Platelet Reactivity Assay

- Whole blood is taken into anti-coagulant
- 35 µl of the blood is then stimulated with agonist under mixing for 5 mins

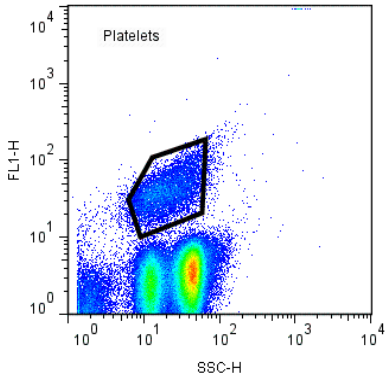


- 5µl is removed, diluted and labelled using a platelet specific antibody to allow single platelet counts to be established
- Remaining sample can be further analysed

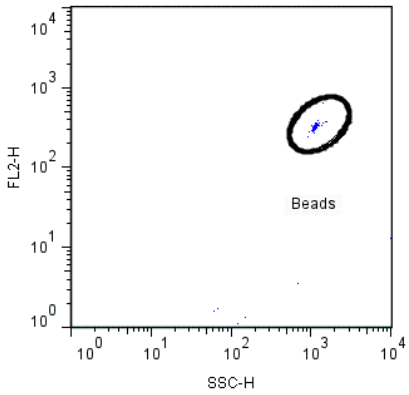
Armstrong et al. Blood. 2015 Sep 3;126(10):e11-8.

Whole Blood Platelet Reactivity Assay

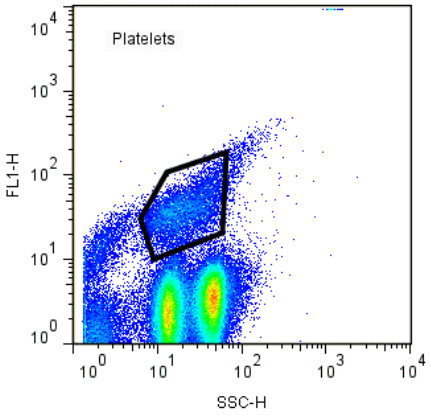
Un-mixed platelet population



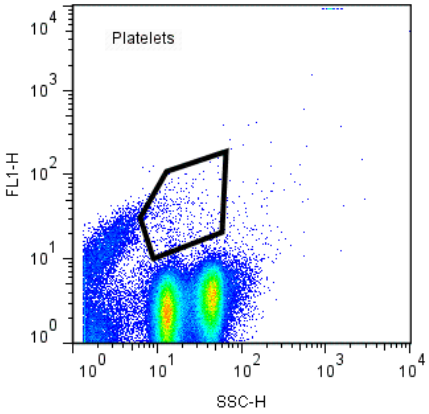
CountBright bead population



Vehicle-mixed platelet population



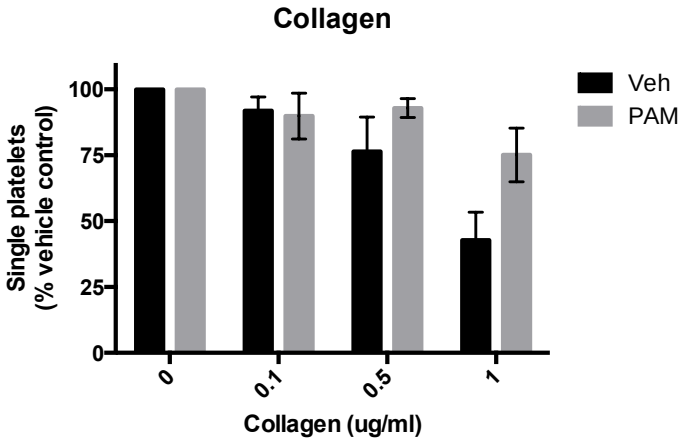
Agonist-mixed platelet population

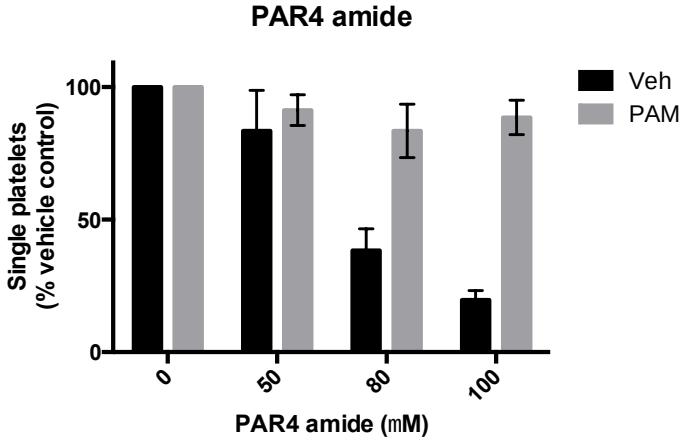


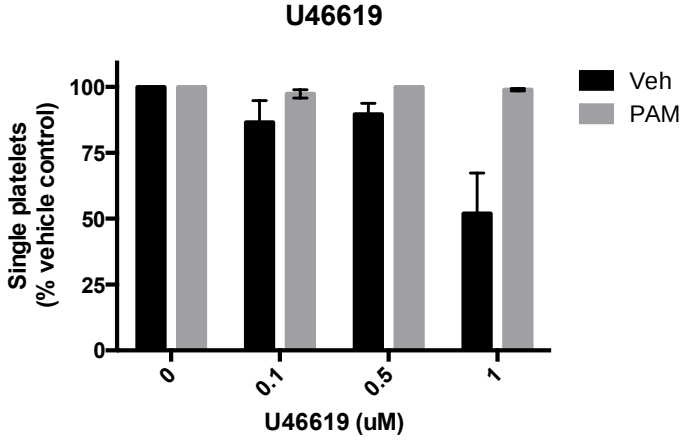
Armstrong et al. Blood. 2015 Sep 3;126(10):e11-8.

Whole Blood Platelet Reactivity Assay

Effect of $P2Y_{12}$ antagonism







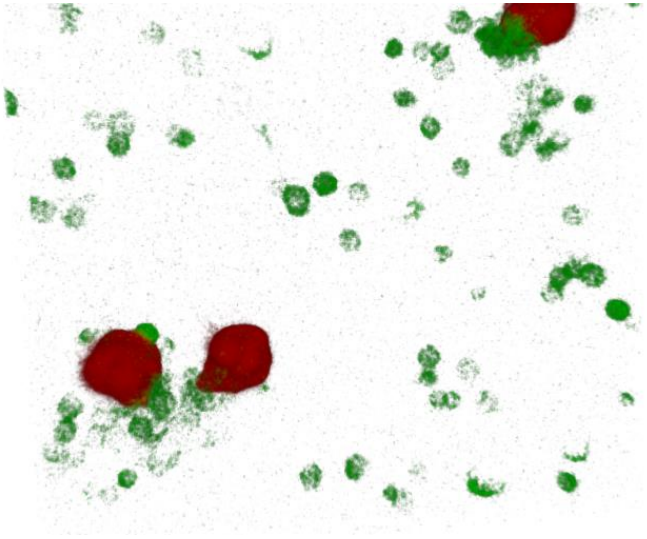
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Whole Blood Platelet Reactivity Assay

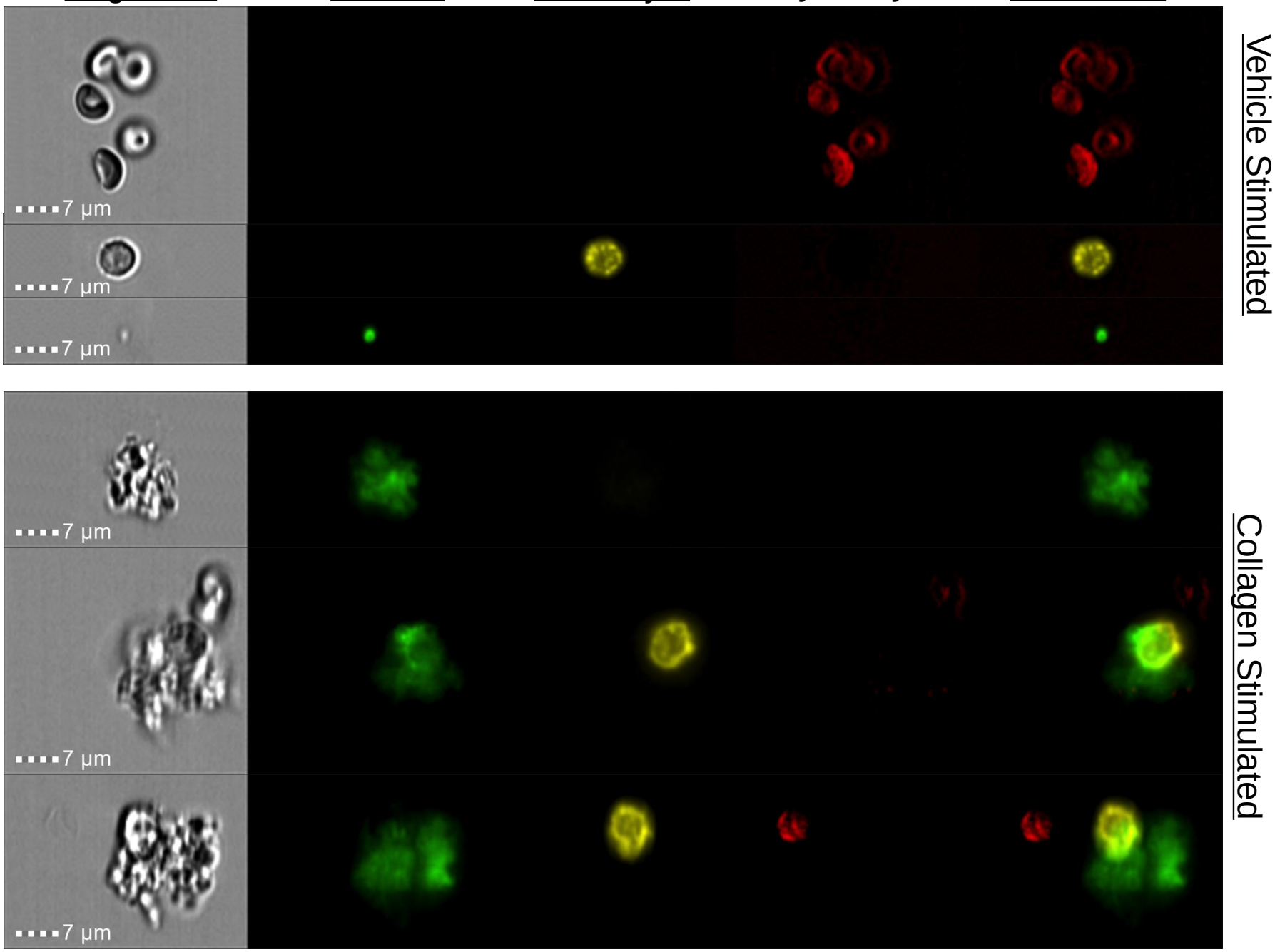
Vehicle Stimulated



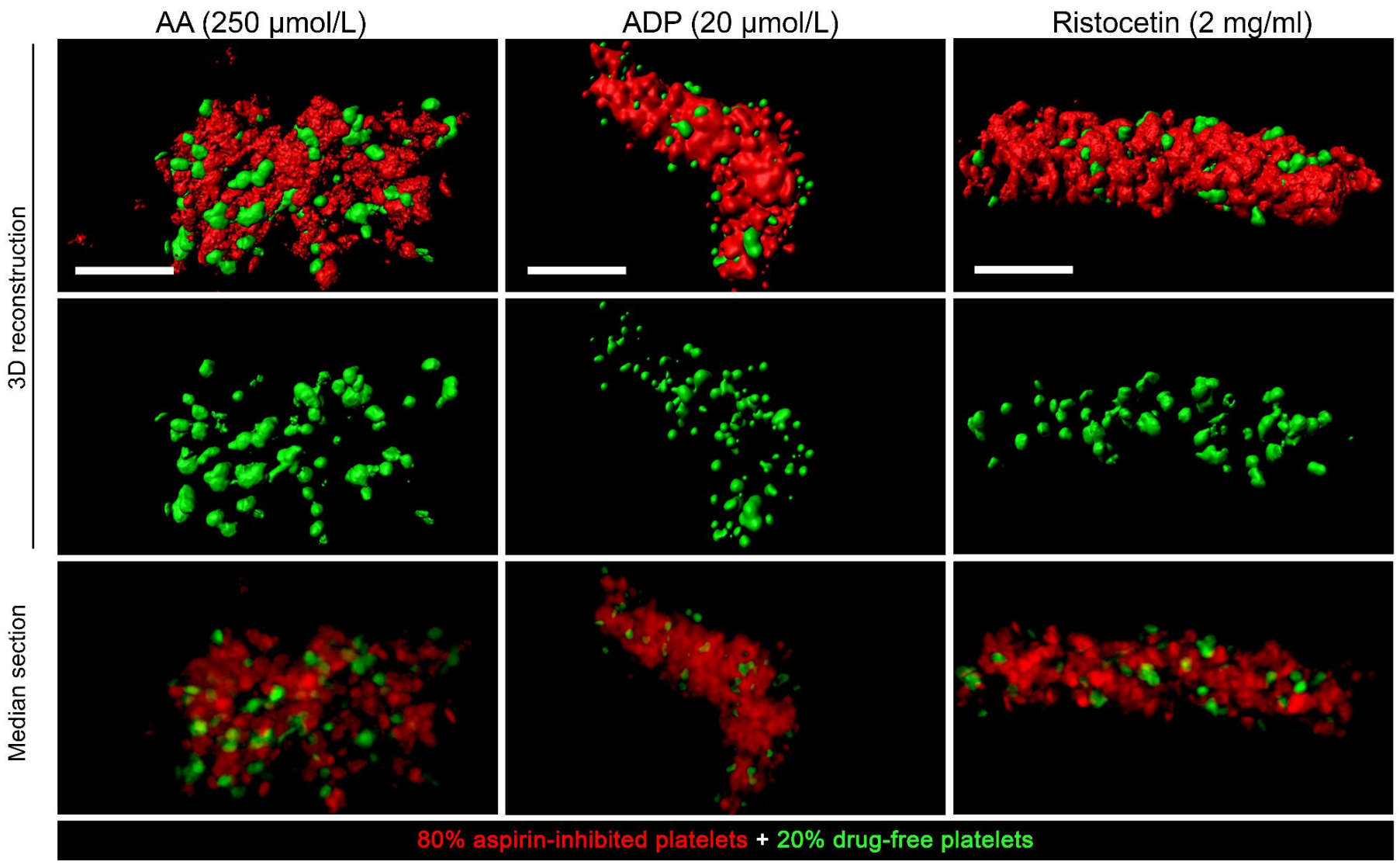
U46619 Stimulated



Armstrong et al. Blood. 2015 Sep 3;126(10):e11-8.


Brightfield
Platelet
Leukocyte
Erythrocyte
Combined
7 µm
7 µm
7 µm
Vehicle Stimulated
7 µm
7 µm
7 µm
Collagen Stimulated

Morphology of aggregates containing 20% uninhibited and 80% aspirin-treated platelets:



Summary

- There are well-established methods for both light transmission aggregometry in platelet rich plasma and whole blood aggregometry and a wealth of literature.
- We have been interested to develop low volume, accessible, and cheap alternatives.
- Two novel methods for investigating platelet aggregation
  - Optimul (in PRP)
  - Whole blood and FACS screening

- Small volumes needed mean that more in-depth testing can be achieved which is relevant for diagnosis and for large cohort analyses. The remaining volume of blood can be used for a broad range of potential additional analyses
  - Sample imaging
  - Flow cytometric analysis of other blood cells – eg: Platelet neutrophil aggregates
  - Proteomic analysis
  - Cyclic nucleotide measurements
  - Releasate measurements (ELISA)

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