The use of flow cytometry to assess platelet function and activation – is it time to throw out the platelet aggregometer??

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Platelets

- Small anucleate discoid cells
- Involved in primary haemostasis:
  - platelet plug formation occurs at sites of injury within seconds
  - important in stopping blood loss from capillaries, small arterioles, and venules
- Internal structure and membrane play central role
Platelet internal structure

- Golgi zone
- Dense granules
- Microtubules
- Trilaminar unit membrane
- Mitochondria
- Dense tubular system
- 
- Lysosomes
- 
- Glycocalyx
- 
- Open canalicular system
- 
- Glycogen granules
- 
- α granules
Platelet reactions in haemostasis

- **Adhesion**: platelets come into contact with endothelium (blood vessel injury)

- **Shape change**: platelets become spherical and extend pseudopodia

- **Secretion**: contents of platelet granules released (e.g. ADP, fibrinogen, etc)

- **Aggregation**: platelets aggregate via specialised receptors

- **Procoagulant activity**: negatively charged phospholipid translocated to outer surface of the platelet membrane and involved in coagulation cascade
Assessment of platelet function and activation

- Platelet count and morphology
- Bleeding time – historical
- Light transmission aggregometry – gold standard
- Rapid, point-of-care assays
Light transmission aggregometry

- Measures increase in light transmission through a platelet suspension when platelets are aggregated by an agonist

- Many variables may affect the results

- Accuracy and reproducibility of technique poor

- Difficult to interpret results in cases of low platelet count
Typical aggregation tracing
Flow cytometry and platelet activation

- **Advantages:**
  - whole blood (physiological, minimal manipulation)
  - minimal volumes required (usually 5 μl per test)
  - used in patients with profound thrombocytopenia
  - measure spectrum of activation-dependent changes
  - quantitative results

- **Disadvantages:**
  - requires a flow cytometer (expensive)
  - dedicated operator
  - must avoid artefactual activation (collection, time delay)
Whole blood flow cytometry of platelets

**Detection of Activated Platelets in Whole Blood Using Activation-Dependent Monoclonal Antibodies and Flow Cytometry**

By Sanford J. Shattil, Michael Cunningham, and James A. Hoxie

*Blood, Vol 70, No 1 (July), 1987: pp 307-315*

### Flow Cytometry Process Diagram

1. Blood
2. Anticoagulant (usually buffered Na Citrate)
3. Dilution
4. Monoclonal antibody/isotype control (labelled with fluorophore)
5. Add agonist
6. Fixation
7. Dilution
8. Analysis
Flow cytometry consensus protocol

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, Stefan Barlage, Diethelm Tschöpe, Kenneth J. Clemetson, Alison H. Goodall, Alan D. Michelson, Alan T. Nurden, T. Vincent Shankey, for the European Working Group on Clinical Cell Analysis

From the Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Germany;
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Thromb Haemost 1998; 79:885-896
Platelet changes studied by flow cytometry
Platelet surface antigen markers

- CD41 – Glycoprotein IIb
- CD42a – Glycoprotein IX
- CD42b – Glycoprotein Ib
- CD61 – Glycoprotein IIIa
Flow cytometric markers of platelet activation

- CD62p (P-selectin) expression:
  - component of α granule membrane
  - mediates adhesion of activated platelets to neutrophils and monocytes
  - circulating degranulated platelets rapidly lose surface P-selectin to plasma pool

- CD63 expression:
  - integral protein of platelet dense granule and lysosomal membranes
Flow cytometric markers of platelet activation

- **PAC-1 binding:**
  - monoclonal antibody directed against fibrinogen binding site exposed by conformational change in GP IIb/IIIa complex of activated platelets

- **Mepacrine (quinacrine) uptake:**
  - taken up by platelet dense granules and emits green fluorescence (FL1)
  - can also measure release after addition of thrombin
  - washing step is required
**Flow cytometric markers of platelet activation**

- **Annexin V binding:**
  - phospholipid binding protein (Ca\(^{2+}\) dependent) which allows detection of PS exposure on outer cell surface of activated platelets (fluorescent-conjugated annexin V)

- **Platelet-leukocyte complexes:**
  - thrombin-activated platelets adhere to isolated fractions of monocytes and neutrophils - mediated primarily through platelet CD62p
  - count CD45/CD42b positive events
Flow cytometric markers of platelet activation

- Platelet microparticles:
  - membrane-bound vesicles derived from platelets which characteristically express PS and also express antigens from cell of origin
  - produced by several mechanisms including activation, mechanical disruption of platelets (e.g. freeze-thaw, CPB), storage, apoptosis
  - demonstrate procoagulant phospholipid activity in functional assays e.g. XACT (Exner et al, Blood Coagul Fibrinolysis, 2003;14:773-779)
Flow cytometric markers of platelet activation

- Platelet vasodilator stimulated phosphoprotein (VASP):
  - intracellular signalling molecule which is non-phosphorylated at basal state and phosphorylated in PGE1 inhibited platelets
  - commercial assay available which measures VASP phosphorylation in the presence of PGE1
  - clopidogrel responsiveness can be demonstrated by the persistence of VASP phosphorylation induced by PGE1 with simultaneous addition of agonist, ADP
Typical platelet scatterplot and histograms
Typical platelet scatterplot and histograms
Typical platelet scatterplot and histograms
Typical platelet scatterplot and histograms
Typical platelet scatterplot and histograms
Platelet CD62p: TRAP activated

Platelet CD62p: resting

Platelet CD62p: TRAP activated
Platelet-leukocyte complexes
Platelet-leukocyte complexes
Platelet-leukocyte complexes
Platelet-leukocyte complexes
Platelet-leukocyte complexes: TRAP activated

Platelet-granulocyte complexes: resting

Platelet-granulocyte complexes: TRAP activated
Platelet microparticles: <1 µm/Annexin V+
Platelet reactivity index (PRI) =

\[
\frac{(\text{MFI}_{\text{PGE1}} - \text{MFI}_{(\text{PGE1} + \text{ADP})})}{\text{MFI}_{\text{PGE1}}} \times 100
\]

Bad responders to clopidogrel 85.8% 6.6% Good responders to clopidogrel
Clinical Examples
Glanzmann’s Thrombaesthenia

- Hereditary disorder – absence of platelet GPIIb/IIIa

- Platelet Count = 159 x 10⁹/L

- Platelet Aggregation
  - Adrenalin: Markedly reduced
  - ADP: Absent
  - Collagen: Absent
  - Ristocetin: Normal
  - Arachidonic Acid: Markedly reduced
Glanzmann’s Thrombaesthenia

- GpIb
- GpIIb
- GpIIIa
- PAC-1
  - TRAP activated

Normal
Glanzmann’s
Bernard Soulier Syndrome

- Hereditary disorder – absence of platelet GPIb/IX
- Platelet Count = 50 x 10^9/L
- Difficult to perform platelet aggregation
Storage Pool Disorder

- **Platelet Count** = $214 \times 10^9$/L

- **Platelet Aggregation**
  - Adrenalin: Reduced
  - ADP: Primary wave only
  - Collagen: Normal
  - Ristocetin: Normal
  - Arachidonic Acid: Normal
Storage Pool Disorder

- Platelet Glycoproteins

GpIb  
GpIX  
GpIIb  
GpIIIa

<table>
<thead>
<tr>
<th>Red</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>SPD</td>
</tr>
</tbody>
</table>
Storage Pool Disorder

- Platelet Activation Markers

CD62p | CD63 | PAC-1

TRAP Activated

Normal

SPD
Storage Pool Disorder

- Mepacrine Staining

![Graph showing MFI vs Mepacrine (µm) with Normal and SPD lines.](image)

- Graph for 6µm Mepacrine

- Normal
- SPD

![Histogram showing FL1-H distribution.](image)
? Scott’s Syndrome

- Hereditary disorder – defective scrambling of membrane phospholipids

- Platelet Count = 389 x 10⁹/L

- Platelet Aggregation
  - Adrenalin: Normal
  - ADP: Normal
  - Collagen: Normal
  - Ristocetin: Normal
  - Arachidonic Acid: Normal
? Scott’s Syndrome

Platelet Glycoproteins

- GpIb
- GpIX
- GpIIb
- GpIIIa

Normal

? Scott’s
**? Scott’s Syndrome**

- **Platelet Activation Markers**

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**CD62p**  
**CD63**  
**PAC-1**  
**Mepacrine**

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TRAP Activated

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Normal  
? Scott’s
Scott’s Syndrome

- Microparticles

Unstimulated

Collagen Stimulated

Ionophore Stimulated
Myelodysplasia

- Platelet Count = 129 x10⁹/L

- Platelet Aggregation
  - Adrenalin: Delayed, but normal
  - ADP: Primary wave only
  - Collagen: Normal
  - Ristocetin: Normal
  - Arachidonic Acid: Normal
Myelodysplasia

- Platelet Glycoproteins

GpIb  GpIX  GpIIb  GpIIIa

Normal  Patient
Myelodysplasia

- Platelet activation markers

CD62p    CD63    PAC-1

TRAP Activated
Myelodysplasia

![Graph showing MFI vs Mepacrine (µm) for Normal and Patient samples. The graph illustrates a comparison between normal and patient samples, indicating differences in MFI values at various concentrations of Mepacrine.]
Coronary stent patients on clopidogrel

- VASP phosphorlyation

![Graph showing VASP Platelet Reactivity Index (%)]

Patients: VASP Platelet Reactivity Index (%)

Normals: VASP Platelet Reactivity Index (%)

p = 0.01
Further data on clinical cases

- Clinical Case Studies (Stream B) Thursday 14th August – David Connor:
  - The Utility of Flow Cytometry to Assess the Cellular Origin of Circulating Procoagulant Phosphatidylserine (3.30 – 3.45pm)
  - Evaluation of the VASP Flow Cytometric Assay for the Assessment of Clopidogrel Responsiveness and Comparison to the Accumetrix P2Y12 Inhibition Assay (3.45 – 4.00pm)
The LATEST in laboratory platelet analysers.....