Applications of flow cytometry in Immuno-haematology

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DECEMBER 4, 2015  HALL-B  14.40-15.00
The end of gating? An introduction to automated analysis of high dimensional cytometry data.

Kumar P.1, Hartmann E.1, Winton D.1, Todorovski V.1, Kasaian D.1, Herberman R.1
Flow cytometry is a method of measuring multiple physical and chemical characteristics of particles by optical means.

- Peripheral blood cells, bone marrow cells, bacteria, yeast

A suspension of single cells or other particles in a suitable buffer, usually PBS.

- Typical density: $10^5 - 10^7$ cells / ml

Incubate Acquire
Parts of a flow cytometer:

- **Optics**
  - Laser
  - Light 488nm

- **Fluidics**

- **Electronics**
  - FL2 PE
  - SSC
  - FSC

- **Computer**
  - FL3 PerCP, Cy5
  - FL1 FITC

Light detectors
5 critical areas in flow cytometry

1. BIOHAZARD PRECAUTION
   - Treat all samples as hazardous
   - Routine addition of bleach (clean cycle)

2. INSTRUMENT SETUP
   - Daily QC (Flow Check & Flow Set)

3. SPECIMEN HANDLING
   - Single cell suspension (no ACD for BM specimens)
   - RT only (16-28 deg C)
   - Lyse-Wash vs Lyse-No Wash

4. REAGENT SELECTION
   - Choice of fluorophores critical
     - For weaker Ag expression, choose brighter colour
   - Colour compensation

5. DATA ACQUISITION
   - 100,000 events or 300 seconds
   - Sample aspirate rate (<1000 events/sec)
Mean Fluorescence Intensity

How Flow Cytometers Work

The fluorescence intensity measured is proportional to the number of fluorescent molecules bound to the cell.

Fluorescent labelled microspheres
Fc-specific
Allows determination of ABC
Presentation and Interpretation of Data

Single parameters can be displayed as a histogram.

Dual parameter data can be displayed in two dimensions using dot, density or contour plots.
Traditional applications of flow cytometry

Determination of lymphocyte subsets in HIV infection
Traditional applications of flow cytometry

Lineage assignment in leukaemias & lymphomas

Multi-parameter flow cytometry $\rightarrow$ MRD/prognosis

Immunophenotype: CD19$^+$ CD5$^+$ CD23$^+$ CD22$^-$ FMC7$^-$ CD79b dim

Chronic Lymphocytic Leukaemia

Cytoplasmic ZAP70$^+$ Surface CD38$^+$ ---- unfavourable clinical course
Applications of flow cytometry in Immuno-haematology

1. Coombs negative AIHA
   - Reticulocyte analysis by flow cytometry
2. Paroxysmal nocturnal hemoglobinuria (PNH)
3. Detection of HbF and RhD for diagnosis of FMH
4. Platelet activation markers in stored platelet concentrates
Coombs-negative AIHA

- AIHA: acquired disorder caused by the production of Abs against RBCs
- The DAT is still the gold standard test for the diagnosis of AIHA
  - Direct antiglobulin test by CTT (Diaclon)
  - Direct antiglobulin test by GT/CAT (Liss-Coombs cards)
- 2-10% of patients with warm AIHA have negative DAT (Coombs test)
- Sensitivity of DAT limited to visual analysis (500 IgG / RBC)
- Flow cytometry can not only detect RBC-bound Ig, but also quantitate the number of Ig molecules on cell surfaces
  - Detection: FITC-conjugated mouse anti-human IgG
  - Quantitation: Quantum Simply Cellular (QSC) beads
Flow cytometry protocol

- RBCs wash x 3 in saline
- Labelled with FITC conjugated goat anti-human IgG
  - Isotype control
- Incubate RT dark 20-30 min
- Cells washed x 2 in cold PBS
- Acquisition done on FC
- Minimum: 10,000 events
Flow cytometry(+) DAT(-) AIHA

Table I. Flow cytometric analysis of AIHA patients.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Hb (g%)</th>
<th>Retic (%)</th>
<th>S.Bil (mg%)</th>
<th>LDH (IU/l)</th>
<th>CTT Poly</th>
<th>IgG</th>
<th>C3d</th>
<th>GT Poly</th>
<th>IgG</th>
<th>C3d</th>
<th>Flow cytometry</th>
<th>MFI</th>
<th>Result</th>
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<tbody>
<tr>
<td>1</td>
<td>22/F</td>
<td>Primary AIHA</td>
<td>6.8</td>
<td>8.5</td>
<td>2.8</td>
<td>2124</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>4+</td>
<td>4+</td>
<td>2+</td>
<td>Pos</td>
<td>19.7</td>
<td>Pos</td>
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<tr>
<td>2</td>
<td>35/F</td>
<td>Primary AIHA</td>
<td>5.1</td>
<td>25</td>
<td>3.8</td>
<td>1817</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>Pos</td>
<td>8.63</td>
<td>Pos</td>
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<td>3</td>
<td>27/F</td>
<td>Primary AIHA</td>
<td>7.5</td>
<td>16</td>
<td>4.9</td>
<td>1618</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>4+</td>
<td>3+</td>
<td>2+</td>
<td>Pos</td>
<td>17.15</td>
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<tr>
<td>4</td>
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<td>Rheumatoid arthritis</td>
<td>9.9</td>
<td>2.9</td>
<td>2.3</td>
<td>690</td>
<td>2+</td>
<td>2+</td>
<td>Neg</td>
<td>3+</td>
<td>2+</td>
<td>Neg</td>
<td>Pos</td>
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<td>Primary AIHA</td>
<td>3.2</td>
<td>20</td>
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<td>1374</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>4+</td>
<td>3+</td>
<td>1+</td>
<td>Pos</td>
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<td>Neg</td>
<td>Neg</td>
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<td>1+</td>
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<tr>
<td>7</td>
<td>31/F</td>
<td>SLE</td>
<td>6.5</td>
<td>2</td>
<td>2.4</td>
<td>834</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1+</td>
<td>1+</td>
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<tr>
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<td>SLE</td>
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<td>Neg</td>
<td>Neg</td>
<td>W+</td>
<td>W+</td>
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<td>20</td>
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<td>1013</td>
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<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>7.1</td>
<td>Pos</td>
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<tr>
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<td>62/F</td>
<td>Rheumatoid arthritis</td>
<td>8.3</td>
<td>9</td>
<td>4.1</td>
<td>678</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>Pos</td>
<td>8.33</td>
<td>Pos</td>
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<td>11</td>
<td>41/F</td>
<td>AIHA follow up</td>
<td>6.9</td>
<td>3</td>
<td>0.9</td>
<td>410</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>3.4</td>
<td>Neg</td>
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<tr>
<td>12</td>
<td>24/m</td>
<td>AIHA follow up</td>
<td>7.5</td>
<td>2.2</td>
<td>0.7</td>
<td>510</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>3.3</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>3.3</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Application of flow cytometry in detection of red cell bound IgG in Coombs negative AIHA

_Hematology_, August 2006; 11(4): 295-300

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1Department of Transfusion Medicine, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India, and
2Department of Haematology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.
Quantitative analysis of RBC bound IgG

QSC beads (Bangs Laboratories Inc., Fisher, USA) ----- used as an external standard
Covalently coated with goat anti-mouse IgG (Fc specific) antibodies
Recognises mouse monoclonal IgG molecules & human Ig molecules on cells

Create a calibration curve
Quantitative analysis of RBC bound IgG

<table>
<thead>
<tr>
<th>Sample (No.)</th>
<th>Tube DAT (Polyspecific IgG+C3d)</th>
<th>Mean fluorescence intensity (MFI)</th>
<th>ABC values (IgG molecules)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4+</td>
<td>114.64</td>
<td>31,725</td>
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<tr>
<td>2</td>
<td>2+</td>
<td>5.75</td>
<td>1,753</td>
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<tr>
<td>3</td>
<td>1+</td>
<td>1.65</td>
<td>524</td>
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<tr>
<td>4</td>
<td>3+</td>
<td>12.87</td>
<td>3,823</td>
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<td>5</td>
<td>trace</td>
<td>0.80</td>
<td>260</td>
</tr>
<tr>
<td>6</td>
<td>negative</td>
<td>0.26</td>
<td>88</td>
</tr>
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<td>7</td>
<td>negative</td>
<td>0.31</td>
<td>104</td>
</tr>
<tr>
<td>8</td>
<td>negative</td>
<td>0.23</td>
<td>78</td>
</tr>
</tbody>
</table>

(Asian Pac J Allergy Immunol 2011;29:364-7)
Non-traditional applications of flow cytometry

Reticulocytes can be distinguished from erythrocytes by their high content of RNA. There are several stains that can be used for RNA, one of them is thiazole orange (Davis and Bigelow, 1994).

Highly fluorescent reticulocytes = Reticulocyte maturity index (RMI)
Paroxysmal nocturnal hemoglobinuria (PNH)

- Acquired chronic haemolytic anemia
  - ?Coombs neg AIHA, refractory anemia, AA
- Clinical manifestations:
  - Pancytopenia (80%)
  - Infection (50%)
  - Hemoglobinuria (20%)
  - Thrombosis (20%)
- Cause: clonal haemopoietic stem cell mutation in PIG-A gene on Chr. X
  - Effect: No GPI anchor on cell surfaces – absent CD55 (DAF), MIRL (CD59) making cells prone to complement-mediated lysis (Alt pathway)
   - Lysis of RBC → hemoglobinuria
   - C3 convertase → Platelet activation, microparticle formation → TF levels → Thrombosis

C3 convertase
Platelet activation, microparticle formation
TF levels
Thrombosis
Diagnosis of PNH

- Traditional assays
  - Sucrose hemolysis (sugar water)
  - Acidified serum (Ham’s) test
  - PNH gel card

False +ve and false –ve
Not useful for small PNH clones (quiescent phase, post trf)
Cannot assess size of PNH clone

How to interpret gel card agglutination
Flow cytometry for PNH

- **Standard Flow cytometry**
  - Greater sensitivity & specificity (1-2% PNH clones)
  - Analysis of non-RBC populations (PNH granulocytes have normal life span, hence greater accuracy of PNH clone size)
  - At least 2 GPI-linked proteins should be absent (ICCS guidelines)
  - Use of one-colour FITC anti-CD55/anti-CD59
A prospective comparison of four techniques for diagnosis of paroxysmal nocturnal hemoglobinuria

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SUMMARY

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder with altered expression of glycosylphosphatidylinositol (GPI)-anchored proteins, resulting in the increased susceptibility of erythrocytes to complement-mediated lysis. This study compared the available laboratory methods for detection of PNH cells and evaluated their utility in routine clinical practice. Fifty patients were evaluated by flow cytometric immunophenotyping (FCMI) using CD55 and CD59 monoclonal antibodies, PNH gel card test (GCT), Ham test and sucrose
Standard flow cytometry protocol for PNH

- EDTA-anticoagulated blood
- For erythrocytes
  - 10μl 1:10 diluted whole blood in PBS
  - 15 min RT with CD59-FITC and CD55-PE
  - Wash, resuspend pellet, analyse
  - Dual negative CD55-CD59- RBC % = PNH clone
- For leukocytes
  - 50μl whole blood with 20μl cocktail
    - CD66b-FITC, CD13-PE, CD45-PerCP, CD14-APC in dark for 15 min
  - Lyse RBC and wash
  - Analyse granulocyte gate (CD45+CD13+CD14- high SSc)
Pitfalls using standard markers

Testing for PNH in Red Blood Cells:

- RBC's with normal CD59 expression (Type I cells)
- clone with complete CD59 deficiency (Type II cells)
- clone with complete CD59 deficiency (Type III cells) and partial CD59 deficiency (Type II cells)
Advanced flow cytometry for PNH

- High sensitivity reagent cocktails/carefully selected conjugates with standardised protocols
- FLAER

Fluorescently labelled inactive variant of aerolysin

Aerolysin: toxin GNB *Aeromonas hydrophila*
Advanced flow cytometry for PNH

Standardising Leucocyte PNH Clone Detection: An International Study

L. Whitby, M. Fletcher, S. Richards, E. Acton, M. Keeney, M. Borowitz, A. Illingworth, R. Sutherland and D. Barnett

Fletcher M etal. In Cytometry B Clin Cytom 2014 [Epub ahead of print]
Feto-maternal haemorrhage

- Detection of FMH and estimation of volume prevents HDFN
  - RhD/ABO incompatibility
  - Invasive prenatal procedures
- Accurate volume estimation allows correct dose administration of Rh-D Ig
- Methods available for detection:
  - Kleihauer-Betke test
    - Problems: Collection method, inter-observer variability
  - KB +ve – proceed to Flow cytometry
  - Other described methods: rosetting (4ml); GAT
- Most sensitive and specific – FLOW CYTOMETRY (gold standard)
Flow cytometry for FMH

- **AIMS:**
  1. Detection of RhD +ve cells in RhD –ve mother
  2. Determine volume of bleed

- Antibody selected should react with all known D phenotypes (except Rh-DVI)
- 50µl of RBC suspension + 5µl of Anti-D FITC + 2.5µl CD45 PerCP (2 tubes) with 0.2% and 1% D+ control
- 500,000 events to be acquired (BCSH guidelines)

Volume of fetal bleed (ml): % fetal cells by flow cytometry (minus background) × 18 × 1.22
Follow-up (BCSH guidelines 1999)

**RECOMMENDATION 10**

A repeat maternal sample should be taken and screened 72 hours after the total dose of anti-D immunoglobulin injection (48 hours if the anti-D Ig was given iv). This is to check for clearance of fetal cells.

**Evidence level lib, Recommendation grade B**

**REPORTING FMH RESULTS TO CLINICIANS**

It is important that FMH results are timely and effectively communicated. This will allow clinicians to manage the woman appropriately.

The report format needs to communicate the following:
- The reason for the sample (e.g. post-delivery, sensitising event, follow-up of significant FMH etc)
- The result of the FMH test in 'mL fetal red cells' rounded to the nearest mL
- Whether any supplementary anti-D immunoglobulin is required if a standard dose of anti-D immunoglobulin has already been administered
Stored platelet concentrates

- Pooled platelets are obtained from random donor (RDP) or single donor (SDP)
- They are kept at RT (20-24degC) on a shaker for maximum 5 days
- Activation during storage may be a cause of reaction following transfusion
- Several QC methods of stored platelet components
  - Cell count
  - pH
  - Swirling
- Role of flow cytometry
  - Markers of platelet activation
  - Redistribution of phosphatidyl serine (PS) from inner membrane surface to exterior
  - Fluorescent-labelled annexin V binds to PS with high sensitivity & specificity
  - Activation of thrombocytes is marked by increased CD62P (P-selectin) expression
1. Live cells
2. Early apoptotic cells
3. Late apoptotic or necrotic cells
Evidence of platelet activation in stored concentrates

Table 2: Platelet activation markers are stored in buffy coat-derived platelet concentrate (BC) and platelet rich plasma-platelet concentrates (PRP-PC).
The future is ........ limitless

The end of gating? An introduction to automated analysis of high dimensional cytometry data.

Rame F1, Hartmann EJ1, Mehdian K1, Truex RC1, Klusman J1, Bochner B1
QUESTIONS