Human Progenitor Cell Enumeration by Flow Cytometry Practical Considerations

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Clinical Flow Cytometry
Objectives

- Conceptual overview: How the Flow Cytometer is able to detect Progenitor Cells
- CAP regulations
- Quality assurance and daily QC of the instrument(s)
- Turn around time
- Gating strategies for rare event analysis
- Correlation and collection efficiency
CD34 enumeration is an Antigen/Antibody Determination

- Governed by the “laws” of Antigen/Antibody interactions.
- Detection of antibody bound by cells is accomplished by fluorescence
Antigen/Antibody Interactions

- Affinity (for antigen) and Avidity (strength of binding) of Antibody.
  - Monoclonal Antibodies

- Incubation times and conditions
  - Direct effect on TAT

- Antibody and Antigen excess “Prozone phenomena”
  - Requirement to optimize Cell and Antibody concentrations
What Antigens are of Interest?

- **CD45**: The common leukocyte antigen.
  - Variably expressed with immature cells (blasts/progenitor cells) expressing it dimly.
  - Mature Lymphocytes and Monocytes express it brightly.

- **CD34**: Defines a family of differentially glycosylated type I transmembrane single chain glycoproteins.
  - CD34 epitopes have been separated in three classes based upon sensitivity to neuramidase and glycoprotease.
  - Clinical assays typically monitor Class III epitope of the CD34 molecule.
Other Antigens of Interest for Allogeneic and CD34 enriched products

- **CD3**: Positive on T Helper/Inducer and T Suppressor/Cytotoxic Lymphocytes subpopulations.
- **CD4**: With CD3 coexpression defines the T Helper/Inducer subpopulation of Lymphocytes.
- **CD8**: With CD3 coexpression defines the T Suppressor/Cytotoxic subpopulation of Lymphocytes.
- **CD19**: Positive on B lymphocytes during most stages of maturation (from Bone Marrow through terminally differentiated = Plasma cells).
- **CD56+/CD16+/CD3-** lymphocytes are NK cells.
- **CD13**: Expressed on Myeloid Cells (Monocytic and Granulocytic).
- **CD14**: Expressed on Mature Monocytic Cells
Flow Cytometry: A **Multiparametric** Approach for Cell Characterization

### Which Parameters?
- **Forward Scatter**: Related to Cell Size
- **90º angle Light Scatter**: Related to Cell Granularity or Complexity
- **Presence of certain Cell Associated Peptides**
  - Detected by Monoclonal Antibodies
  - These Antibodies are directly conjugated to any one of a number of Fluorochromes
- **Often overlooked**: Time
Light Scatter Gating

Scale
- 1000
- 200
- 100
- 50
- 40
- 30
- 20
- 15
- 8
Flow Cytometry: Fluidics

The use of focused light (lasers) to interrogate cells delivered by a hydrodynamically focused fluidics system.
Optics - Light Scatter

Fluorescence Detectors

Fluorescence detector (PMT1- PMT5)
Fluidics Quality Control

- Daily/periodically
  - Very bright beads are run through the cytometer to verify fluidics and laser alignment.
  - Half-peak C.V.s must be less than 2.0
Fluorochrome Emission Spectra
Anatomy of a Flow Cytometer
PMT Optimization

- Daily/periodically
  - Very dim beads are run through the cytometer to verify PMT voltages applied for each fluorochrome.
  - Monitor Mean Fluorescence Intensity.
  - Any adjustment in PMT voltages must be accompanied by verification of compensation.
Compensation of Fluorochromes Due to Spectral Overlap

5 C 1 L Compensation Settings

<table>
<thead>
<tr>
<th></th>
<th>FL1</th>
<th>FL2</th>
<th>FL3</th>
<th>FL4</th>
<th>FL5</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1</td>
<td>16.8</td>
<td>36.1</td>
<td>6.3</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>FL2</td>
<td>0.9</td>
<td>1.9</td>
<td>3.5</td>
<td>0.3</td>
<td>1.7</td>
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<tr>
<td>FL3</td>
<td>3.5</td>
<td>15.2</td>
<td>4.7</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>FL4</td>
<td>6.3</td>
<td>31.7</td>
<td>0.7</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>FL5</td>
<td>1.7</td>
<td>6.5</td>
<td>17.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>
**PMT Optimization/Compensation QC**

**Examples:**
- Unacceptable Compensation (left)
- Acceptable Compensation (right)
Progenitor Cell Analysis = Rare Event Analysis

- Normal individuals have low levels of circulating CD34+ Progenitor cells (1-3/µl)
- Boolean (Sequential) gating is imperative to correctly identify the population of interest.
CD34 Enumeration = Rare Event
Analysis: ISHAGE (Boolean) Gating

- FLO.30592: Are sequential (Boolean) gating techniques used to define the CD34+ stem cells?
- FLO.30564: Is there a procedure in place to document CD34 cellular viability, where applicable?
- 7AAD is a fluorescent compound (peak emission = 675nm) which is excluded from viable cells (nonviable cells are permeable).
- Note: In single platform determinations (where count beads are incorporated) a logical gate to exclude the beads from the viability is required.
CD34 Enumeration = Rare Event
Analysis: ISHAGE (Boolean) Gating

- The second gate is used to distinguish WBCs (CD45+).
- Red blood cells (including nucleated RBCs) are CD45-.
- This histogram is gated such that only viable events (Not count beads AND 7AAD negative) are evaluated.
CD34 Enumeration = Rare Event
Analysis: ISHAGE (Boolean) Gating

- The third gate focuses on CD34+ events.
- The only events evaluated in this histogram are viable WBCs.
  - Not Count Beads
  - 7AAD negative
  - CD45+
CD34 Enumeration = Rare Event
Analysis: ISHAGE (Boolean) Gating

- The fourth gate examines the CD45 staining intensity of the CD34+ events.
- Progenitor cells are CD45 dim to moderate. Therefore CD45 bright events should be excluded from this gate.
- The events evaluated in this histogram are viable CD34+ cells.
  - Not Count Beads
  - 7AAD negative
  - CD45+
  - CD34+
CD34 Enumeration = Rare Event Analysis: ISHAGE (Boolean) Gating

- **Whole Viable cells**
  - Not pieces/parts of cells.
  - Gated on:
    - Not count beads
    - Viable cells
    - CD45+
    - CD34+
    - CD45 dim to moderate

- **Linked histograms:**
  - Gated to:
    - Not count beads
    - Viable cells
    - CD45+
CD34 Enumeration = Rare Event Analysis: ISHAGE (Boolean) Gating

- The histogram depicted to the right is part of the protocol for two primary reasons:
  - To qualitatively verify that compensation settings are appropriate
  - To create a COUNT BEAD region which is excluded from the VIABLE gate and included in the CAL factor histogram which follows.
Count beads, when uniformly resuspended, are standardized to yield a known (CAL Factor) number of beads/µl.

Fluidics can be monitored over time with the CAL factor region.

“Single Platform” Cell counts (CD34+ cells/µl) are normalized based on the CAL Factor.
Rare event analysis by FCM: How Many are Enough?

- FLO.30585: Are a statistically valid number of CD34+ events collected to ensure clinically relevant precision and accuracy?

- \( r = \left(\frac{100}{CV}\right)^2 \), where \( r \) is the number of events meeting the required criterion

- can be used to determine that size of the database that will provide a given precision

- Example CD34 desired C.V. = 10%
  
  \[
  r = \left(\frac{100}{10}\right)^2 \\
  r = 10^2 \text{ or } 100 \\
  \text{S.D.} = \sqrt{\text{target events counted}}
  \]
Calculation of the total number of events required for a given precision

<table>
<thead>
<tr>
<th>For a CV of (%):</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td># of positive events to be recorded:</td>
<td>10000</td>
<td>1600</td>
<td>400</td>
<td>100</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>When occurring at a frequency of:</th>
<th>Total # of events which must be collected *:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(%)</td>
<td>1:n</td>
</tr>
<tr>
<td>Total # of events which must be collected *:</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.0x10^5</td>
</tr>
<tr>
<td>1</td>
<td>1.0x10^6</td>
</tr>
<tr>
<td>0.1</td>
<td>1.0x10^7</td>
</tr>
<tr>
<td>0.01</td>
<td>1.0x10^8</td>
</tr>
<tr>
<td>0.001</td>
<td>1.0x10^9</td>
</tr>
</tbody>
</table>

* The number of events to be collected which are >10^6 would require considerable acquisition times; i.e. at a flow rate of 5000/sec, it would take ~3.5 min to observe 10^6 events.
CD34 Progenitor Cell Analysis: How Many are Enough?

- Stops are set according to the following:
  - Either 100 CD34+ events or
  - 100,000 Viable WBCs or
  - 10 minutes acquisition/tube. (Whichever occurs first)

- Practical consideration: At OSU the clinical decision to perform apheresis is made when the patient’s peripheral CD34 count is 10 CD34+ cells/µl or above.
Human Progenitor Cells: QC Process Control

- Stabilized blood products with an established range of CD34+ cells.

- FLO.23800: CAP Requirement to report 2 levels:
  - 1 Level chosen to represent results expected from majority of products.
  - 1 Level chosen to fall below Clinical Decision Value (For OSUMC this value is 10 CD34+ cells/µl)
**Process Control: Status Flow Pro (High Level)**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>% Total WBCs (Range)</th>
<th>Number/μL (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>0.564</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td>(0.299 - 0.829)</td>
<td>(12.3 - 34.0)</td>
</tr>
</tbody>
</table>

---

**Legend:**

- 1: STEM CELL STATUS FLO PRO CD34, PRO, Status Flow Pro, FC038-34, CD34+, X, % Cited
- 2: STEM CELL STATUS FLO PRO CD34, PRO, Status Flow Pro, FC038-34, CD34 COUNT, X, Cells/μL
Low level control is prepared daily by diluting the CD34 stabilized product 5 fold in “normal” (no CD34+ cells) peripheral blood to achieve the target value of > 10 CD34+ cells/µl.

Acceptable normal ranges are established in house for each lot of CD34+ control product.
# Process Control: Status Flow Pro (Low Level)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>% Total WBCs (Range)</th>
<th>Number/µl (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>0.112 (0.052 - 0.182)</td>
<td>4.62 (3 - 8)</td>
</tr>
</tbody>
</table>
### Process Control: Status Flow Pro (Low Level)

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>Value</th>
<th>Setting</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/15/2008</td>
<td>7:54</td>
<td>0.54</td>
<td>22</td>
<td>flowlab</td>
</tr>
<tr>
<td>3/15/2008</td>
<td>7:58</td>
<td>-</td>
<td>0.13</td>
<td>6 flowlab</td>
</tr>
<tr>
<td>3/16/2008</td>
<td>7:49</td>
<td>0.56</td>
<td>22</td>
<td>flowlab</td>
</tr>
<tr>
<td>3/16/2008</td>
<td>7:52</td>
<td>D</td>
<td>0.14</td>
<td>RPT OK (Bead capture not set)</td>
</tr>
<tr>
<td>3/16/2008</td>
<td>7:55</td>
<td>-</td>
<td>0.18</td>
<td>8 flowlab</td>
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<tr>
<td>3/17/2008</td>
<td>7:22</td>
<td>0.53</td>
<td>20</td>
<td>flowlab</td>
</tr>
</tbody>
</table>
CD3 Enumeration Using a Modified ISHAGE Protocol
CD3 Lymphocytes on CD34
Enriched Products
T Cell Subsets on CD34 Enriched Products

Total Number of Events (WBCs) 80304
Number of Events in (G): 44
44/80304*100 = 0.05% CD4+/CD3+
T Cell Subsets on CD34 Enriched Products

Number of Events in (I):
28

Total Number of Events (WBCs):
80304

28/80304*100 = 0.03% CD8+/CD3+
Validation of CD34 enriched Determinations

- Requires a population of cells that are 100% CD34+
- Mixing studies with normal peripheral blood having a known CD4+/CD3+ count and a known CD8+/CD3+ count will help establish reportable range of CD34 (up to 100%) and CD4+/CD3+ or CD8+/CD3+ down to approaching 0.
Turn-around Times (TAT) for Progenitor Cell Enumeration

- FLO.30571 CAP Requirement CD34 results reported within 4 hours of collection/receipt.
- OSU average TAT is approximately 60 minutes from the time of receipt.
  - Recall Antibody Incubation 15 minutes
  - RBC Lysis 10 minutes
  - Acquisition of 100,000 nucleated events or 10 minutes x 3 tubes (potentially 30 minutes)
Use of CD34 determinations for Collection Efficiency Monitoring

Collection Efficiency = \[
\frac{\text{Peripheral Blood CD34 Count} \times BVP}{\text{CD34 Count Apheresis Product} \times \text{Volume}}
\]

Autologous HPC-A Collection Efficiency By Disease

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>64%</td>
<td>60%</td>
<td>53%</td>
<td>49%</td>
<td>50%</td>
</tr>
<tr>
<td>APL</td>
<td>49%</td>
<td>50%</td>
<td>50%</td>
<td>49%</td>
<td>50%</td>
</tr>
<tr>
<td>HD</td>
<td>33%</td>
<td>39%</td>
<td>42%</td>
<td>50%</td>
<td>41%</td>
</tr>
<tr>
<td>MM</td>
<td>42%</td>
<td>40%</td>
<td>41%</td>
<td>50%</td>
<td>41%</td>
</tr>
<tr>
<td>NHL</td>
<td>40%</td>
<td>35%</td>
<td>35%</td>
<td>46%</td>
<td>45%</td>
</tr>
<tr>
<td>AMYLO</td>
<td>35%</td>
<td>35%</td>
<td>42%</td>
<td>46%</td>
<td>46%</td>
</tr>
<tr>
<td>MEDULL</td>
<td>72%</td>
<td>50%</td>
<td>35%</td>
<td>50%</td>
<td>46%</td>
</tr>
<tr>
<td>Average</td>
<td>42%</td>
<td>42%</td>
<td>42%</td>
<td>42%</td>
<td>42%</td>
</tr>
</tbody>
</table>

Goal > 40%
Use of CD34 determinations for Collection Efficiency Monitoring

Here collection efficiency is used to document instrument (apheresis unit) bias (or lack thereof)
CD34 Correlations when PB Count >10/µl

Peripheral Absolute CD34+ Cell Count Versus Collection Yield (CD34+ x 10^6/kg)
Autologous HPC-Apheresis January - June 2007

95.5% of collections with peripheral absolute CD34+ cell count > 10 collected ≥ 1.0 x 10^6 CD34+/kg

R^2 = 0.944
CD34 Correlations when PB Count ≤ 10/µl

Peripheral Absolute CD34 Cell Count Between 2 - 25
Versus Collection Yield (CD34+ X 10⁶/kg)
Autologous HPC-Apheresis January - June 2007

Mean collection yield when peripheral absolute CD34+ cell count <10 = 0.54 x 10⁶ CD34+/kg
Acknowledgments

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- Rhonda Kitzler and Becky Pearson
- Rachel Bennet
- Lynn Apel and Ira Johnson
Questions?