Poster #

CD34+ STEM CELL ENUMERATION WITH T-CELL EVALUATION: A COMPARISON BETWEEN MANUAL AND AUTOMATED ANALYSIS METHODS

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Introduction

BACKGROUND: Flow cytometry (FCM) is the method of choice to enumerate CD34+ stem cells (SC) in samples for hematopoietic stem cell transplant (HSCT). The ISHAGE protocol is considered the standard method, but like most FCM protocols that rely on manual gating strategies, it suffers from subjective errors due to manual gating.

METHOD: We used 612 Patient Stem Cell data files representing 204 patients. The FCS file sets were analyzed separately using the ISHAGE manual analysis method and probability state modeling (PSM) -automatic analysis method. We also tested the reproducibility of both methods by having multiple trained analysts workup the same set of 15 files. Additionally, 269 files (patients and control) were analyzed for T cell enumeration using both methods.

RESULT: We observed excellent correlation between the manual ISHAGE analysis method compared to the automatic PSM method (R^2= 0.99 SC/ul, R^2= 0.99 SC/WBC). The automated PSM method was 100% reproducible from operator to operator (CV=0, SD=0), while variability increased with the ISHAGE manual analysis method (CV= 0.8-7.6, SD=79.6). Similarly, the two methods had very good correlation in quantifying T cells (r^2=0.92 CD3+/Lymphocytes, r^2=0.94 CD4+/Lymphocytes)

ISHAGE Method

- Uses complex gates
- Uses manually defined regions
- Uses manually positioned regions

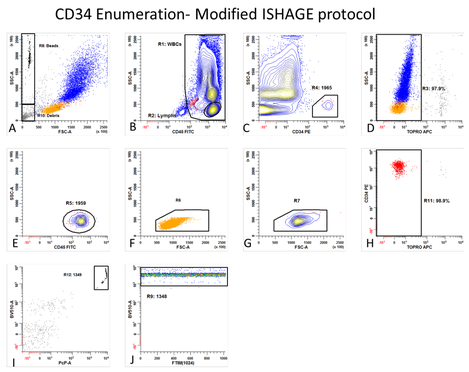

Figure 1: Manual CD34 enumeration strategy based on the ISHAGE Single platform protocol

A: SSC vs. FSC to remove debris (R10) and define beads (R8).
B: CD45 FITC vs. SSC excluding R8 & R10 to define leukocytes (R1) and lymphocytes (R2).
C: CD34 PE vs. SSC to grossly define SC (R4).
D: TO-PRO-3 vs. SSC gated on R1 to resolve viable from non-viable leukocytes.
E: CD45 vs. SSC gated on R1, R3 and R4 to better define SC by excluding CD45 dim platelets (R5).
F: SSC vs. FSC gated on R2 and R3 establishing the scatter characteristic of viable lymphocytes (R6).
G: SSC vs. FSC gated on R1, R3, R4 and R5; with R7 linked to R6 to exclude from SC any monocytes which may have dim CD34 expression associated with bound platelets. Events falling within R7 define the final number of viable SC.
H: TO-PRO-3 vs. CD34 PE gated on R1, R4, R5 and R7 to calculate SC viability (R11).
I: BV510 vs. PCP-A to further define beads (R12).
J: F-TIME vs. BV510 gated on R8 and R12 showing the stability of the sample acquisition over time.

Manual T-Cell Analysis

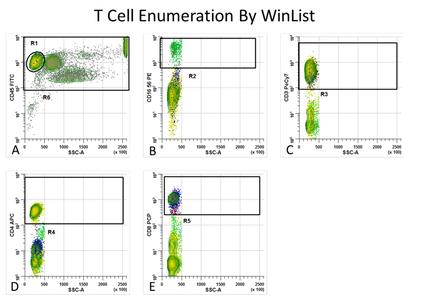

Figure 2: Lymphocyte subset panel using manual gating strategy

A: SSC vs. CD45 FITC to define lymphocytes (R1). B, C, D and E are all gated on R1 and define NK cells (R2: SSC vs. CD16/56), T cells (R3: SSC vs. CD3), CD4 helper T cells (R4: SSC vs. CD4), CD8 cytotoxic T cells (R5: SSC vs. CD8), respectively.

GemStone Analysis

GemStone uses a patented Probability State Modeling (PSM) system to locate and classify populations.

Expression profiles are created for each marker relevant to the population of interest and grouped into a "Cell Type."

The PSM system automatically positions each Expression Profile to select events belonging only to that cell type. Multiple cell types are used in event classification.

Two Expression Profiles

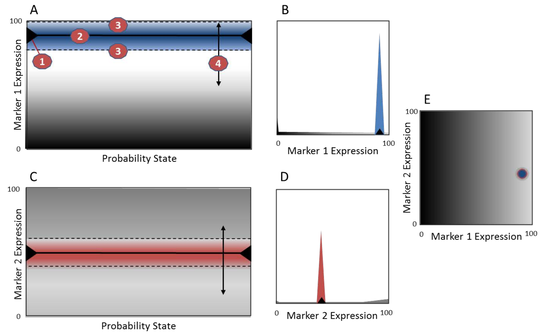

Figure 3: GemStone Expression Profiles relate to Classic Histograms

This cartoon features two GemStone Expression Profiles (panels A and C) shown with more traditional 1-parameter (panels B and D) and a 2-parameter histogram (panel E) of the same data. These demonstrate the relationship between the Expression Profiles and classic histograms.

Note that in panel A the peak position (1) is identified by small black triangles. GemStone has a peak finder system that automatically finds relevant peaks. The mean of the peak is represented by the solid black line (2). The boundaries of the 95 percent confidence limits are delineated by the dashed lines (3). The black double arrow (4) has been added to the cartoon to show the range that the automated Peak Finder system is allowed to shift to find a peak of interest. In B and D the peak position is marked by the back triangles and the co-expression of marker 1 and 2 is illustrated in E

The Expression Profiles serve to define the cell types and model the intensity differences of the events within a sample. The Probability State organizes the events in some biologic order based on the marker expression. For example, bright CD34 expression is expected in immature cells while more differentiated cells lose CD34 expression, which in GemStone would be modeled as a step down. Here, Constant Expression profiles were used exclusively in the CD34+ Stem Cell Model because we selected for events that had relatively the same intensity of expression for each marker.

The event selection process is designed to define commonly used phenotypes but does so without the hard boundaries that are imposed in gating analyses.

GemStone CD34+ Stem Cell Model

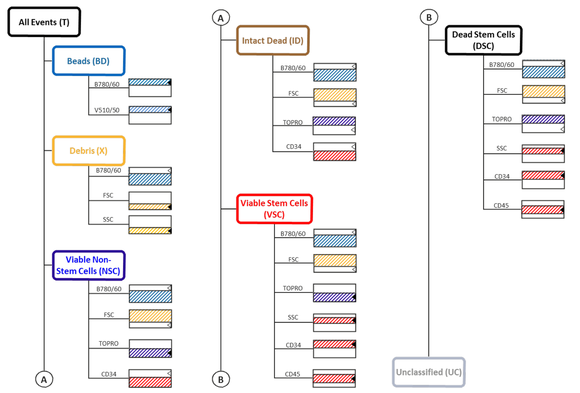

Figure 4: The CD34+ Stem Cell model comprises six Cell Types. Each Cell Type is defined using multiple Expression Profiles (EP)

Automated classification (selection) of events starts with the Bead Cell Type (BD). The Bead EPs are automatically adjusted by the software for the optimal position based on criteria in the model. Since the Bead Cell Type is the first Cell Type in the model the EP positions are based on all events in the file.

The model defines six different cell types that probabilistically categorizes the events. The cell types are Bead (BD), Debris (X), Viable Non-stem Cells (NSC), Intact Dead (ID), Viable Stem Cells(VSC), and Dead Stem Cells (DSC). Many of the cell types are designed so that they not only attract specific events but repel others. This attract/repel characteristic of many of the cell types greatly reduces false positive and negative categorizations.

Once all of the EPs in all Cell Types have been optimized, all events are passed through the model again and competitively re-classified (assigned) to one cell type. Each event can only be assigned to one Cell Type. Any event not selected by one of the Cell Types is marked as Unclassified. This process is completely autonomous and performed without any user interaction.

GemStone T-Cell Model

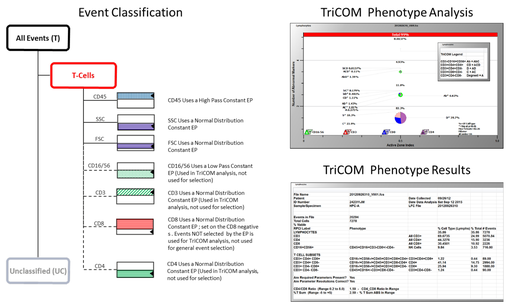

Figure 5: T-Cell Model and TriCOM

The T-Cell model used with the CD34+ Stem Cell Analysis starts with Expression Profiles (EP) that select for CD45+, low Side Scatter and low Forward Scatter events to identify the cell type of interest (lymphocytes). Subsequent Expression Profiles are used to obtain the percentages of specific subsets of these events using GemStone's TriCOM feature.

Using information from Expression Profiles for CD16/56, CD3, CD8 and CD4, TriCOM categorizes events based on whether they are within, below or above the specific EP marker. Because these Expression Profiles have the "selection" feature disabled, all three states can be considered and used for phenotype analysis and enumeration.

A comprehensive set of phenotype results are displayed in the information box on the patient report.

Manual vs. Automated Results

CD34+ Stem Cells

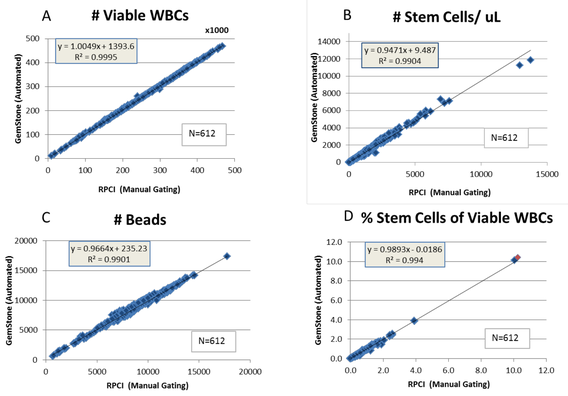

Figure 6: Correlation of results for #Stem Cells/uL

Panel A: The results for estimating the number of live WBC are highly correlated between GemStone and manual analysis (r2 = 0.99). Panel B: the correlation for the number of SC/uL (r2 = 0.99). Panel C: the correlation for the percentage of SC to WBC (r2 = 0.99). Panel D: the correlation for the percentage of SC to WBC (r2 = 0.99). N= 612 for all correlations.

T-Cells

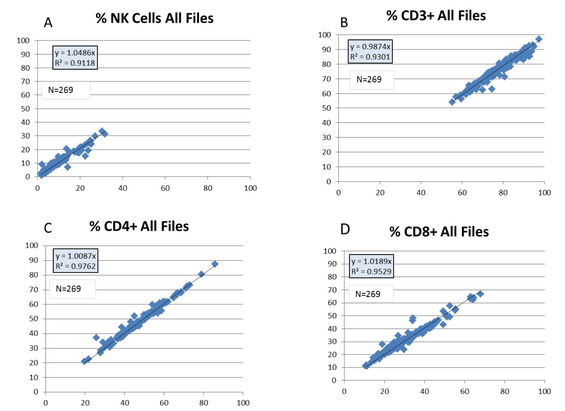

Figure 7: Correlation of results for T-Cells

Each panel is showing the percentage of positive events of the total lymphocyte count for 269 files. Panel A: the correlation between the two methods for the percentage of CD56/16+ NK cells (r2 = 0.91). Panel B: the correlation for the percentage of CD3+ (r2 = 0.93). Panel C: the correlation for the percentage of CD4+ T cells (r2 = 0.97). Panel D: the percentage of CD8+ T cells (r2 = 0.95).

Reproducibility

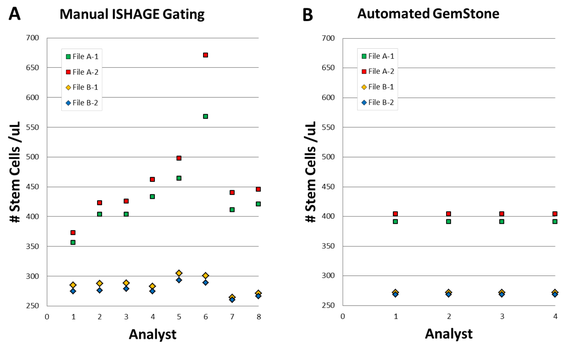

Figure 8: GemStone results are 100% reproducible between analysts

The results shown are for the Number of Stem Cells per Microliter obtained from the analyses of the same four data files. The files represent two cases (A and B) with two replicates (1 and 2). The data were analyzed by several different well-trained analysts using either a manual ISHAGE gating method or the automated GemStone PSM method.

Panel A depicts the typical result variation when the same files are analyzed manually using the established ISHAGE gating method. The coefficient of variation for number of viable stem cells per micro liter when done by the manual meth for ranged from 0.8 to 7.6. In Panel B the GemStone results are the same for all analysts. A total of 15 files were analyzed in this manner with similar results. In every case, the automated GemStone method gave identical results for every analyst, while the manual method produced varying result values.

Conclusions

PSM is a validated analysis method to enumerate CD34+ stem cells and T cells; it is well correlated with the ISHAGE Single Platform method, and more importantly, it has excellent reproducibility from operator to operator. Using the automated PSM method has also met our goals of reducing technical time needed and eliminating repeat analysis due to mistakes in region placement.

Discussion

After performing this study and running a number of cases in parallel the GemStone automated method was adopted as our method of choice for analysis. Built-in quality assurance flags available through the GemStone software allowed the technical staff to quickly identify outlier results caused by typical technical errors and take fast and efficient remedial action

Some of the key components for development and implementation of the PSM method were:

- A good set of data representing a full range of patient samples and controls
- Rigorous instrument QC and care to maintain antibody staining intensity performance from lot to lot of reagents
- Documentation of how the manual analysis was performed
- Ability to create a database of manual and PSM results for easy comparison
- Identification and review of outliers using the database
- Ability to test models with full automation enabled us to fine tune the model and obtain the optimal correlation while maintaining absolute reproducibility throughout the study.

The PSM system of analysis was fully implemented in the Clinical laboratory running 4 or 5 stem cell analyses per day plus controls in Oct 2014 without any difficulties encountered to date. The walk away batch analysis, automation and internal QC flags incorporated into the data analysis routines permits accelerated reporting and rapid identification of problematic samples.

References

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