

Enumeration and Viability of Nucleated Cells from Bone Marrow, Cord Blood, and Mobilized Peripheral Blood

Introduction: Bone Marrow, Cord Blood, Stem Cell Transplantation

Worldwide, more than 25,000 allogeneic hematopoietic cell transplants are performed each year. These utilize stem cells isolated from bone marrow, peripheral blood, and cord blood. For many years, allogeneic transplantation has been effective in treating several types of cancer, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndromes (MDS), non-Hodgkin's lymphoma (NHL), chronic myeloid leukemia (CML), and Hodgkin's lymphoma (HL). In addition, allogeneic transplantation is being used to treat several non-malignant disorders, including severe aplastic anemia (SAA), plasma cell disorders, and inherited immune and metabolic disorders.

With improved HLA matching and advances in post-transplant support, survival rates are improving each year. Unrelated donor transplant outcomes are now comparable to related donor outcomes for several patient populations. With increased awareness, expanding donor databases, and advances in transplant techniques, the annual number of allogeneic transplants is continuing to grow. In 2010, approximately 60% of all allogeneic hematopoietic cell transplants utilized peripheral blood stem cells, involving a less invasive collection procedure than traditional bone marrow harvesting. Approximately 20% of transplants involved bone marrow and 20% utilized cord blood. Pediatric transplants continue to rely more heavily on bone marrow and cord blood, in part due to smaller sample requirements.

With the growing number of options for transplantation, a universal method for accurate analysis of a wide variety of cell and sample types is essential. In this application note, we present a simple, accurate image cytometry method for determination of nucleated cell concentration and viability in bone marrow, cord blood, and peripheral

blood at various stages of processing utilizing data generated in collaboration with AllCells, a market leader for primary cells.

Sample Collection and Cell Processing Methods

Bone Marrow: Bone Marrow (BM) was drawn into a 60cc syringe containing heparin (80 U/mL of BM) from the posterior iliac crest, 25mL/site, from a maximum of four sites. The whole BM was diluted with phosphate-buffered saline. Samples were analyzed within 2 hours of collection. Mononuclear cells (MNC) were isolated from BM aspirate using density-gradient separation. CD34+ stem/progenitor cells were isolated from MNC fraction using immunomagnetic microbead separation.

Cord Blood: Cord Blood (CB) was drawn from the umbilical vein postpartum into heparin and analyzed within 48 hours of collection.

Mobilized Peripheral Blood (mPB) MNC: A healthy donor was injected with 7.0ug to 9.0ug/kg/day of G-CSF for five consecutive days to stimulate the release of CD34+ cells into the blood stream. Apheresis was performed using the COBE Spectra apheresis machine on Day 5 for collection of leukocytes. MNC were isolated from leukapheresis product using density-gradient separation. CD34+ stem/progenitor cells were enriched from mPB MNC using immunomagnetic microbead separation.

Traditional Cell Viability Methods

Many labs processing samples for allogeneic transplantation rely on the traditional methods of staining with Trypan Blue and manually counting cells on a hemacytometer. Alternatively, 7-AAD fluorescence is characterized flow cytometrically, to determine cell concentration and viability. Trypan Blue and 7-AAD are membrane exclusion dyes that

1. National Marrow Donor Program, August 2, 2012, http://marrow.org/Physicians/Unrelated_Search_and_Transplant/Trends_in_Allo_Transplants.aspx

stain non-viable cells possessing compromised membranes. While these methods work fairly well for homogeneous samples, such as CD34+ stem/progenitor cell samples, there are drawbacks to these methods when analyzing heterogeneous samples, such as BM aspirates and CB, where red blood cells (RBC) can be incorrectly represented as viable MNC resulting in an over-estimation of MNC viability.

In this study, we demonstrate a dual-fluorescence method for identification of total nucleated cells and dead nucleated cells in samples contaminated with platelets and RBC. This method was used to analyze BM samples (fresh aspirates, MNC, and CD34+ cells), CB, and mPB samples (MNC, and CD34+ cells).

In some laboratories, total nucleated cell count is performed on fresh samples with a high percentage of RBC and platelets using a lysis/staining procedure. As part of this study, manual total nucleated cell (TNC) counts were compared using 3% acetic acid lysis/Methylene Blue nuclear staining and automated AO/PI nuclear staining analysis without lysis. Unlike the AO/PI dual-staining method, traditional lysis methods cannot measure cell viability.

Experimental Procedure

Primary Reagents:

- Fresh whole bone marrow (AllCells, Part# ABM001)
- Cryopreserved Bone marrow mononuclear cells (AllCells, Part# ABM007F)
- Fresh bone marrow, CD34+ cells (AllCells, Part# ABM009)
- Fresh whole cord blood (AllCells, Part# C-CB101-20ML)
- Fresh mobilized peripheral blood mononuclear cells (AllCells, Part# mPB001)
- Fresh mobilized peripheral blood CD34+ cells (AllCells, Part# mPB009)
- 0.4% Trypan Blue Stain (Mediatech, Part# 25-900-CI)
- Cellometer ViaStain™ AO/PI (acridine orange / propidium iodide) Staining Solution (Nexcelom Bioscience, Part# CS2-0106-5ML)
- 3% acetic acid with Methylene Blue (Stem Cell Technologies, Part# 07060)
- 1X PBS

Equipment:

- Cellometer Vision Cell Analyzer or Cellometer Auto 2000 Cell Viability Counter
- Table-top vortex
- Precision pipettor to dispense 20 μ l

Determination of Cell Concentration and Viability

The Cellometer Vision Cell Analyzer and Cellometer Auto 2000 Cell Viability Counter feature a pre-optimized dual-fluorescence viability assay for a wide variety of primary cell types, including PBMCs, stem cells, and total nucleated cells. For this experiment, we tested viability using Trypan Blue as well as a dual-fluorescence AO/PI method. The AO/PI method is highly recommended for samples containing debris or RBC. We also compared a manual Methylene Blue method (with lysing) and

automated AO/PI method (without lysing) for determination of total nucleated cells.

AO/PI Viability Method

Acridine orange (AO) dye stains DNA in the cell nucleus of all cells to obtain a total nucleated cell count. Propidium iodide (PI) DNA-binding dye is used to stain dead cells and determine cell viability. Healthy cells with intact membranes are impermeable to the PI dye. Dead cells stained with both AO and PI fluoresce red due to fluorescence resonance energy transfer (FRET). Live nucleated cells fluoresce green and dead nucleated cells fluoresce red. Because Cellometer AO/PI live and dead cell counts are conducted in the fluorescent channels and debris/non-nucleated cells do not fluoresce, there is no interference from debris or non-nucleated cells.

AO/PI Viability Staining and Analysis Procedure

1. Dilute fresh bone marrow aspirate, cord blood, and peripheral blood samples with 1X PBS
2. Mix sample well by pipetting up and down at least ten times.
3. Combine 20 μ l of sample and 20 μ l of AO/PI dye solution and mix well by pipetting up and down
4. Load 20 μ l of sample into the disposable Cellometer Counting Chamber
5. Allow cells to settle in chamber for 1 minute
6. Insert Counting Chamber into the Cellometer
7. Select the AO/PI Viability Assay
8. Enter your Sample ID and a Dilution Factor of 2
9. Optimize the Focus and Click Count



The Cellometer Auto 2000 Cell Viability Counter features a user-friendly touch-screen with pre-set assays for primary cell analysis.

Trypan Blue Viability Method

The Trypan Blue dye enters cells with compromised membranes, making them appear dark upon bright field imaging. This dark color enables the Cellometer software to identify and count dead cells independently of live cells. In the bright field / trypan blue method, size differentiation is used to exclude platelets and debris from cell counts, but red blood cells may be counted.

Trypan Blue Viability Staining and Analysis Procedure

1. Mix sample well by pipetting up and down at least ten times.
2. Combine 20 μ l of sample and 20 μ l of trypan blue dye solution and mix well by pipetting up and down
3. Load 20 μ l of sample into the disposable counting chamber
4. Allow cells to settle in chamber for 1 minute
5. Insert Counting Chamber into the Cellometer
6. Select the Trypan Blue Assay
7. Enter your Sample ID and a Dilution Factor of 2
8. Optimize the Focus and Click Count

3% Acetic Acid / Methylene Blue Staining and Analysis Procedure

1. Mix sample well by pipetting up and down at least ten times.
2. Combine 25 μ l of sample and 475 μ l of acetic acid/Methylene Blue solution and mix well by pipetting up and down
3. Perform manual count with a hemacytometer

Experimental Results

AO/PI Viability Results

In order to validate the Cellometer Auto 2000 Cell Viability Counter for primary cell analysis, more than 100 samples from AllCells were tested using the Cellometer Auto 2000. Either Trypan Blue and AO/PI staining methods were compared or Trypan Blue staining was used to compare manual and automated counting. A table listing all of the sample types tested with AllCells and representative images for several sample types are shown below. The bright field images demonstrate the varying degree of sample complexity at different stages of processing. Nucleated cells, RBC, platelets, and debris are visible in bright field images. Only nucleated cells are visible in the dual-fluorescence images. Live nucleated cells are circled in green. Dead nucleated cells are circled in red.

Sample Source	Sample Type	Comments
Bone Marrow	fresh	
	MNC, fresh	Mononuclear cell fraction
	CD34+ frozen	Stem/progenitor cells
Cord Blood	Multiple Myeloma, MNC, fresh	Multiple Myeloma patient sample
	fresh	
	CD34+ frozen	Stem/progenitor cells
	CD34-depleted, frozen	Mononuclear cells depleted of stem/progenitor cells
	MNC, fresh	Mononuclear cell fraction
Peripheral Blood	Mobilized & non-mobilized, fresh	
	MNC, Mobilized and non-mobilized, fresh	Mononuclear cell fraction
	CD34+, frozen and fresh	Stem/progenitor cells
	CD14+, frozen	Monocytes
	CD19+, frozen	B-Cells
	CD56+, frozen	Natural Killer Cells
	SLE, MNC, fresh	Systemic Lupus Erythematosus patient sample

Bone Marrow Aspirate (fresh)

Bone marrow aspirate contains red blood cells (erythrocytes) white blood cells (leukocytes), and platelets (thrombocytes). As seen in the fluorescent image below in which only nucleated cells are visible, nucleated white blood cells make up a very small percentage of total cells in the aspirate sample.

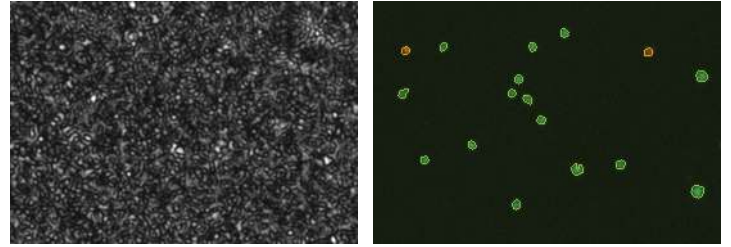


Image 1 and 2 Bright field and dual-fluorescence counted image for a bone marrow aspirate.

Bone Marrow MNC Sample following Density Gradient Separation (cryopreserved)

The majority of platelets and red blood cells are removed upon density gradient separation. The amount of remaining red blood cell contamination will vary from sample to sample. A cell concentration/viability analysis method that is not affected by red blood cell contamination is recommended at this stage of sample processing. As shown by the circles in the images below, non-nucleated cells do not appear in fluorescent images used for Cellometer AO/PI cell counting.

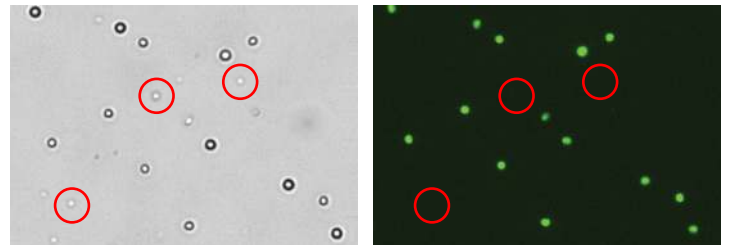


Image 3 and 4 Bright field and dual-fluorescence counted image for bone marrow mononuclear cells following density gradient separation.

Bone Marrow CD34+ Sample following Density Gradient Separation and Immunomagnetic Separation (fresh)

Following immunomagnetic separation, samples contain little to no red blood cell contamination. A simple trypan blue viability method or dual-fluorescence method can be used at this stage.

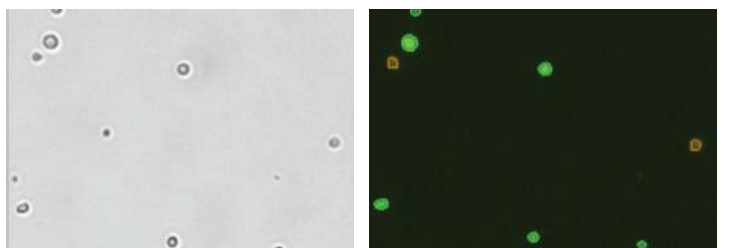


Image 5 and 6 Bright field and dual-fluorescence counted image for CD34+ cells from bone marrow following density gradient separation and immunomagnetic separation.

Cord Blood Sample (fresh)

Similar to bone marrow aspirates, fresh cord blood samples contain a large number of platelets and red blood cells. Dual-fluorescence is required to obtain an accurate cell count and viability determination.

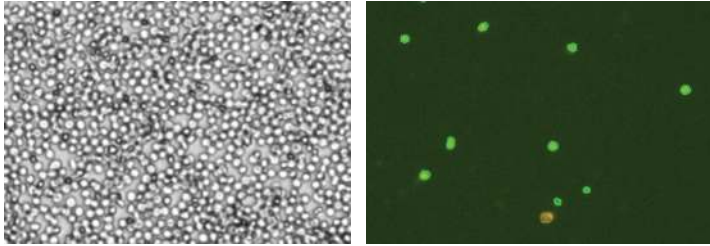


Image 7 and 8 Bright field and dual-fluorescence counted image for a fresh cord blood sample.

Normal Peripheral Blood MNC (fresh)

In the normal peripheral blood MNC sample, there is still evidence of low-level contamination of non-nucleated cells (blue circles). These cells would be counted as nucleated white blood cells in a manual counting system.

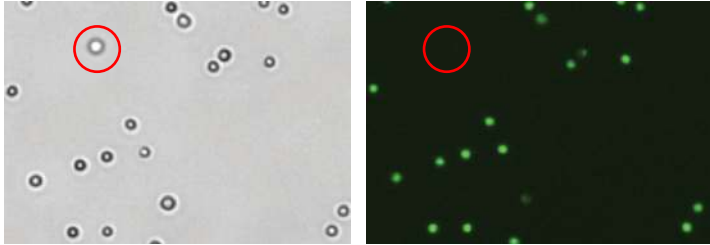


Image 9 and 10 Bright field and dual-fluorescence counted image for a normal peripheral blood sample following density gradient separation.

CD34+ Mobilized Peripheral Blood Sample (fresh)

CD34+ mobilized peripheral blood samples showed very uniform staining of nucleated white blood cells. No interference from non-nucleated red blood cells was observed. A simple trypan blue viability method or a dual-fluorescence viability method could be used at this stage.

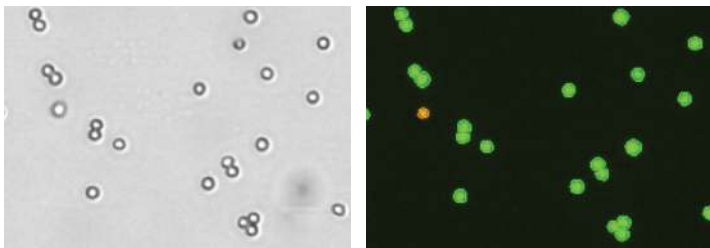


Image 11 and 12 Bright field and dual-fluorescence counted image for a CD34+ mobilized peripheral blood sample.

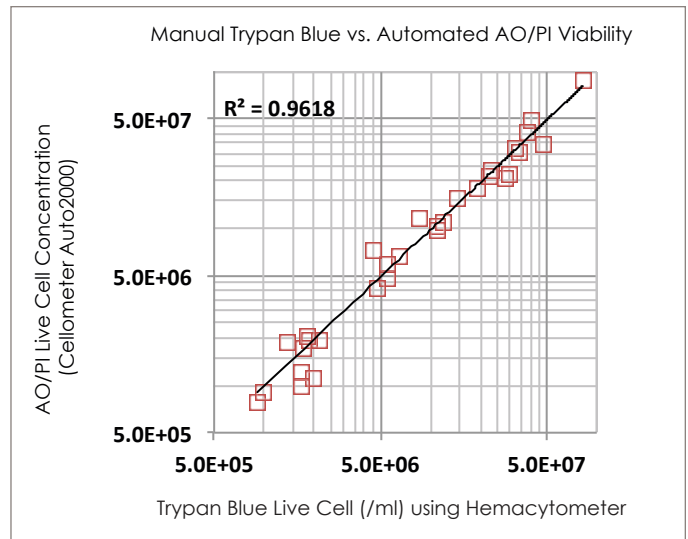
The number, concentration and percent viability of nucleated cells measured for representative samples is listed in the table above right. For each sample, 20 μ l was loaded into the Cellometer Counting Chamber and four areas of the counting chamber were automatically imaged and counted. The total number of nucleated cells counted, the concentration of nucleated cells in the original sample, and the percent viability were automatically generated by the Cellometer system.

Sample*	Cell Population	Count (# of cells)	Concentration (cells / mL)	% Viability
Bone Marrow Aspirate (fresh)	Total	1,456	5.06 x 10 ⁶	99.7%
	Live	1,460	5.05 x 10 ⁶	
	Dead	4	1.4 x 10 ⁴	
Bone Marrow MNC	Total	988	3.44 x 10 ⁶	92.6%
	Live	985	3.43 x 10 ⁶	
	Dead	4	1.38 x 10 ⁴	
Bone Marrow CD34+ (fresh)	Total	500	1.74 x 10 ⁶	92%
	Live	460	1.61 x 10 ⁶	
	Dead	40	1.39 x 10 ⁵	
Cord Blood (fresh)	Total	276	9.57 x 10 ⁵	91.3%
	Live	252	8.74 x 10 ⁵	
	Dead	24	8.32 x 10 ⁴	
Normal Peripheral Blood MNC	Total	1,656	5.73 x 10 ⁶	91.3%
	Live	1,512	5.24 x 10 ⁶	
	Dead	144	4.97 x 10 ⁵	
Mobilized Peripheral Blood	Total	2,474	9.33 x 10 ⁶	92.6%
	Live	2,475	8.84 x 10 ⁶	
	Dead	199	6.90 x 10 ⁵	

*All samples tested were from different donors.

Trypan Blue vs. AO/PI Viability Results

Thirty processed samples (via density gradient separation and/or immuno-magnetic separation) were tested using both manual Trypan Blue and automated AO/PI viability methods. There was a very high degree of correlation between the two methods. (R² value of 0.96%).



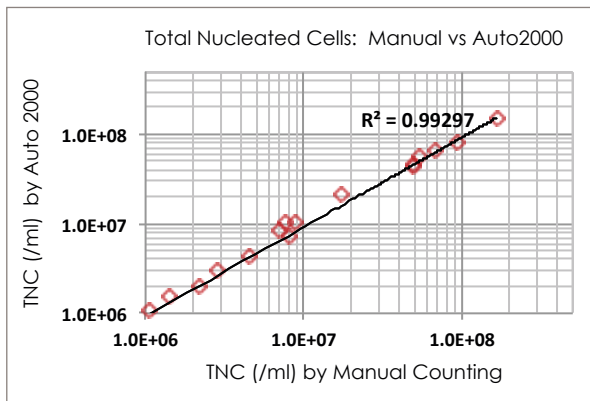
The six samples displaying the largest discrepancy in percent viability were the bone marrow MNC samples and CD34-depleted bone marrow samples containing varying amounts of RBC contamination. Trypan Blue viability ranged from 89 to 98% and AO/PI viability ranged from 40 to 71% for these samples. Manual counts included RBC in total cell counts, resulting in higher percent viability calculations. Purified samples with no red blood cell contamination, including

CD34+ cells, showed excellent correlation between trypan blue and AO/PI.

Due to the density of cells and degree of non-nucleated cell contamination in the fresh cord blood, bone marrow aspirate and peripheral blood samples, they could not be tested using the trypan blue viability method. A dual-fluorescence method is highly recommended for analysis of fresh patient samples.

3% Acetic Acid/Methylene Blue vs. AO/PI Total Nucleated Cell Results

Sixteen samples (normal and mobilized peripheral blood, cord blood, and bone marrow aspirates) were lysed/stained with 3% acetic acid/Methylene Blue. Cells were counted manually using a hemacytometer, one count (four quadrants) per sample. A second aliquot of each sample was stained with AO/PI without lysing. Live and dead nucleated cells were counted automatically using the Cellometer Auto 2000. There was excellent correlation between total nucleated cells counted using the two methods (R^2 value of 0.99%).



Conclusion

A wide variety of sample types were successfully evaluated for cell concentration and viability using a single AO/PI dual-fluorescence staining method. The Auto 2000 includes pre-set assays for analysis of fresh bone marrow, cord blood, and peripheral blood samples, processed samples with varying

degrees of RBC contamination, and purified CD34+ samples, enabling accurate, reproducible analysis with minimal training. A correlation study between manual Trypan Blue viability with automated AO/PI viability and manual Methylene Blue total nucleated cell count with AO/PI total nucleated cell count were successfully completed.

Switching to the AO/PI method for fresh bone marrow, cord blood, and peripheral blood samples enables total nucleated cell (TNC) counts and viability characterization of fresh samples that is not possible with the historical methylene blue testing method for TNC calculation. Switching to the AO/PI dual-fluorescence method following density gradient separation eliminates potential over-estimation of cell viability caused by RBC contamination and counting, improving the accuracy of sample characterization.

Automated analysis can decrease test time by as much as 80%, eliminate the inter-operator variability associated with manual counting, and significantly cut training time for new technicians. Archived images and automated data reports provide a permanent QC record for each sample tested. Customized reports can be printed directly from the Cellometer software. Researchers can utilize the AO/PI or Trypan Blue viability methods with the Cellometer Auto 2000 Cell Viability Counter to accurately evaluate the viability of cells upon receipt from vendors like AllCells and to evaluate samples at various stages of processing in their own laboratory.

Product Information

For more information on human primary cells, contact AllCells at 1-888-535-3444, e-mail sales@allcells.com or visit www.allcells.com.

For more information on the Cellometer ViaStain AO/PI Staining Solution (Part# CS2-0106-5ML) the Cellometer Auto 2000 Cell Viability Counter, or the Cellometer Vision Cell Analyzer call Nexcelom at 978-327-5340, e-mail info@nexcelom.com, or visit www.nexcelom.com.

Auto 2000