Determining Cell Concentration by Direct Volume on the BD Accuri™ C6

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White Paper

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Abstract

Technology to quickly and precisely determine the absolute number or concentration of cells with a given phenotype is highly useful in fields such as cell biology and drug discovery. Historically, laboratories performed such counts using a light microscope and hemocytometer, but this method is slow and prone to error. Flow cytometry enabled the rapid identification of phenotypes, but counting the cells required the addition of counting beads to the sample (single platform) or the separate determination of total cell count by hemocytometer or hematology analyzer (dual platform).[1] The BD Accuri™ C6 flow cytometer is a compact, digital, low-cost instrument whose unique fluidics system, driven by peristaltic pumps, can report sample volume directly. This brings the advantages of single-platform flow cytometric cell counts into the hands of small labs and individual researchers.

The work presented here explores several different cell counting applications using the BD Accuri C6: viable cell enumeration in cultured cell lines; immune cell concentrations in human peripheral blood; and platelet counts in whole, unlysed human blood. Each experimental report focuses on methods of sample preparation and data analysis that help to maximize counting accuracy. The data illustrates the advantages of the direct volume method.

The study results suggest that cell concentrations reported with the BD Accuri C6 are highly correlated with concentrations measured using bead counts and are more precise than hemocytometer counts. The direct volume method offers the added benefits of increased speed and reduced cost and complexity. The technology is useful in applications as diverse as, but not limited to, viability of cultured cell lines and human platelet counts.

In studies comparing cell counts between different conditions, either direct volume or counting beads should produce precise, valid comparisons as long as each is used consistently. Since counts by the two methods may differ, however, it may be wise to validate using both methods in studies where the absolute cell concentration is important.

Introduction

The ability to accurately and precisely report concentrations of cell subpopulations is essential in many areas of biological research. For example, changes in viable cell concentrations are important in assessing cell culture integrity, cell-mediated cytotoxicity, and drug toxicity.[2,3,4] In typical experiments, researchers want to compare changes to cell concentrations across a variety of conditions, so results must be determined reliably across a broad range of concentrations. When a large number of samples are compared, the cost and time required for each sample become crucial.

Historically, life science research laboratories performed cell counts using a light microscope and a hemocytometer. A viability dye such as trypan blue allows the viable cells to be identified and counted. However, this method is slow and prone to error. If the target cell subpopulations must be identified phenotypically, a flow cytometer is usually added. However, this only results in multiplication of errors. A better solution would eliminate the hemocytometer and use the flow cytometer to report cell concentration as well.

Digital flow cytometers with laminar-flow fluidics allow fast, phenotypic data collection (up to 10,000 events per second) on a wide range of cell types (sub-micron sized bacteria through large mammalian cell lines). However, because they cannot directly report sample volume, counting beads must be added to each sample to calculate cell concentrations. Since the bead concentration is known, the cell concentration can be calculated based on the ratio of beads to cells.

On the other hand, flow cytometers with syringe-driven fluidics can determine cell concentrations directly, without counting beads. However, they are often limited by lower data acquisition rates (<1,000 events per second), diminished fluorescence and light-scatter resolution, and a propensity for clogs in the flow cell.

The BD Accuri C6 (Figure 1) is a digital flow cytometer with unique laminar-flow fluidics driven by a peristaltic pump system (Figure 2). By monitoring the pressure in the Sample Introduction Probe (SIP), a microprocessor can determine the sample flow rate. This arrangement combines the advantages of hydrodynamically focused cell sampling (high data acquisition rates, good light-scatter and fluorescence resolution) with the ability to accurately report sample volume and automatically report concentrations for any identified population in a sample.

Figure 1. The BD Accuri C6 flow cytometer system.

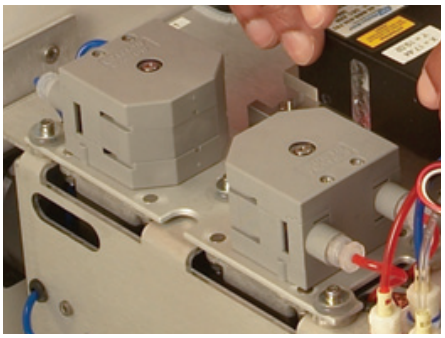


Figure 2. The BD Accuri C6 peristaltic pump system.

The unique laminar-flow fluidics system of the BD Accuri C6, which is driven by peristaltic pumps, combines the advantages of hydrodynamically focused cell sampling with the ability to report absolute cell counts for any identified population in a sample.

Objectives

This paper explores methods of preparing and analyzing samples for determination of cell concentration across several applications: viable cell counts in cultured cell lines, T- and B-cell counts in human peripheral blood, and platelet counts in whole human peripheral blood. The three experiments highlight important factors for accurately determining cell concentrations—such factors are discussed in *blue type*—and illustrate where the direct volume method on the BD Accuri C6 has advantages over other techniques. For comparison, cells were also counted by counting beads and, in one experiment, by hemocytometer.

Materials

Cell lines

Product Description	Vendor	Catalog Number
U937 human lymphoma	ATCC	CRL-1593.2
Jurkat human T-cell leukemia, Clone E6-1	ATCC	TIB-152
3T3 mouse fibroblast	ATCC	CRL-1658

Antibodies

Marker	Fluorochrome	Clone	Vendor	Catalog Number
CD3	FITC	UCHT1	BD Biosciences	555332
CD19	PE	HIB19	BD Biosciences	555413
CD41	PE	HIP8	BD Biosciences	555467

Ancillary reagents and materials

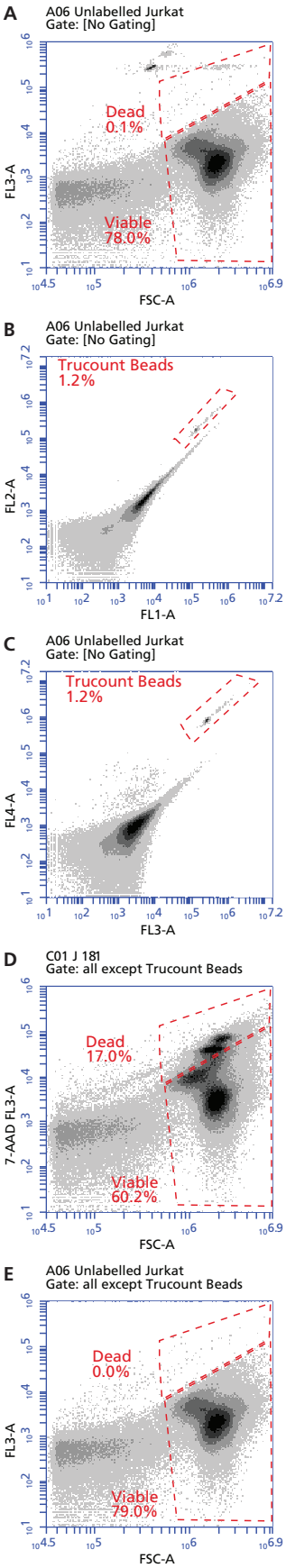
Product Description	Vendor	Catalog Number
BD FACS™ lysing solution	BD Biosciences	349202
BD Pharmingen™ 7-AAD staining solution	BD Biosciences	559925
BD Trucount™ absolute counting tubes	BD Biosciences	340334
Trypan blue	HyClone	SV300840

Instruments

All cytometer data was acquired using a BD Accuri C6 flow cytometer system. Prior to data collection, the instrument was validated for accurate volume recording using BD Trucount tubes, which contain a known number of fluorescent beads in a lyophilized pellet. The acceptable range of bead counts on the BD Accuri C6 is ±20% of the expected value.

BD Accuri™ C6 software was used for acquisition, analysis, and calculation of cell counts. Unless otherwise noted, 25 µL of each sample was collected from the same tube three times using the Medium fluidics setting (flow rate = 35 µL/min, core size = 16 µm). *The 25-µL collection amount was chosen to minimize acquisition time, avoiding sample settling while still collecting a significant number of cells. Researchers can adjust this balance for each experiment, depending on the sample size desired and the propensity for samples to settle.*

To resuspend cells, the samples were flicked several times before placing them on the cytometer. When automating data collection with the BD CSampler™ accessory, use the agitate function to reduce settling.[5] Determine optimal conditions empirically with each sample.



To provide comparative counting bead data, BD Trucount absolute counting tubes were used for all experiments. A known number of beads in the tube were diluted in a known volume (in this case, 500 µL) to determine the expected bead concentration. Counting the beads in the sample then yields the volume, which can be used to calculate the concentration of cells sampled. Bead counts were calculated using an FL1 vs FL2 plot. The comparative counting bead results are discussed under "**Direct volume vs counting beads**" later in this paper.

Microscopic cell counts were obtained using a Neubauer cell counting chamber.

Methods and results

Viable cell counts

Cultures of U937, Jurkat, and NIH/3T3 cell lines were incubated under standard conditions for 7–8 days or 13–14 days, then harvested and resuspended in culture media. From each culture, 0.5 mL was transferred into three BD Trucount tubes for flow cytometry analysis, either undiluted or diluted 1:5 or 1:10 in phosphate-buffered saline (PBS). Five microliters of a 1 mg/mL stock solution of 7-Amino-actinomycin D (7-AAD) solution was added to each sample tube and mixed thoroughly. 7-AAD is a fluorescent viability dye that, like trypan blue, marks cells with compromised outer membranes. Tubes were kept at room temperature (RT) in the dark, gently mixed, and sampled 5–30 minutes after 7-AAD was added. For each cell line, an additional tube of undiluted cells (without 7-AAD) served as an unstained control sample to determine the viable cell gate.

Samples were analyzed on the BD Accuri C6 by setting up a density plot of forward light scatter (FSC) vs 7-AAD fluorescence (7-AAD FL3) in BD Accuri C6 software. A negative control sample for each cell type (without 7-AAD) was used to define the viable-cell gate in that plot (Figure 3A). This gate included events with high FSC and defined the FL3 background fluorescence.

When using BD Trucount tubes, counting beads should be excluded from analysis since they can overlap with the samples and interfere with accurate counts. BD Trucount beads are most accurately identified in a biparametric fluorescence plot such as FL1 vs FL2 (Figure 3B) or FL3 vs FL4 (Figure 3C). Use a plot in which they do not overlap with other samples. In this case, the gate in Figure 3B was used to exclude the beads from the FSC vs FL3 plots (Figures 3D and 3E).

For comparison, undiluted samples were also counted on a hemocytometer. Trypan blue was added 1:10 to undiluted cells in 1.5-mL microfuge tubes. Ten microliters of labeled cells were transferred to the hemocytometer slide, and counts were performed in triplicate. At least 50 non-blue (viable) cells were counted for each replicate.

Figure 3. Viable and dead cell gate placement.

A. *Unstained control cells were used to place the viable cell gate on an FSC vs FL3 plot. BD Trucount beads were identified on* ***(B)*** *FL1 vs FL2 and* ***(C)*** *FL3 vs FL4 plots.* ***D.*** *The bead gate from Figure 3B was used to exclude the beads from the FSC vs FL3 plot.* ***E.*** *7-AAD⁺ cells were used to place the dead cell gate on the FSC vs FL3 plot.*

Figure 4 and Table 1 show the results of counting viable cells using the direct volume method on the BD Accuri C6 compared to the hemocytometer. The mean concentration of viable cells was similar as reported by both methods, with no significant differences found (compared by paired Student's t-test). However, reports were more precise (indicated by standard deviation or %CV) using the direct method, reaching significance in one of three populations (compared by F-test).

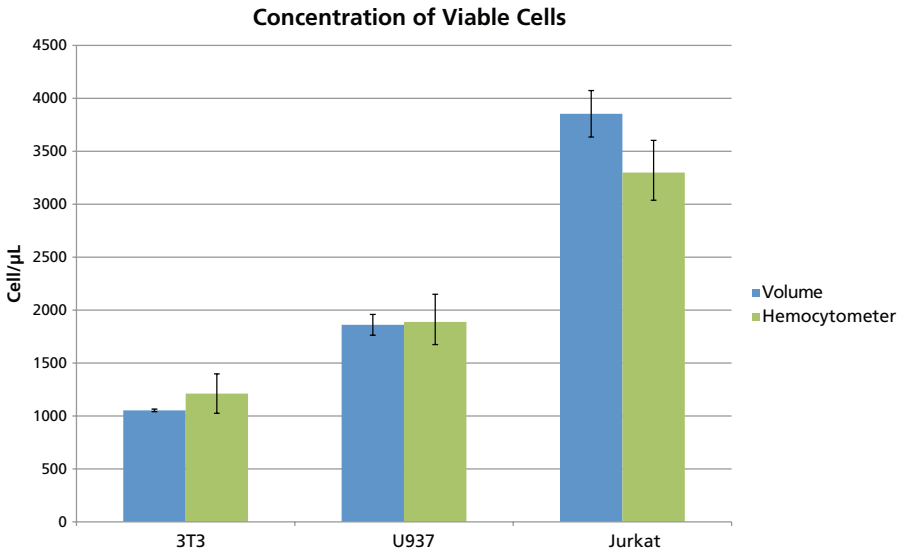


Figure 4. Selected viable cell concentrations by two counting methods.

Comparison of mean viable cell counts per microliter (colored bars) and standard deviations (error bars) determined by direct volume and hemocytometer methods using three different cell lines one week after passage to a new flask.

Table 1. Viable cell concentrations from three cell lines, calculated by direct volume and hemocytometer.

	Cytometer			Hemocytometer			Comparisons	
	Mean (cells/µL)	Std Dev	%CV	Mean (cells/µL)	Std Dev	%CV	Mean (Paired Student's t-Test)	Std Dev (F-Test)
3T3	1,053.0	12.5	1%	1,211.7	186.1	15%	0.254	**0.009**
U937	1,861.3	98.1	5%	1,888.3	261.4	14%	0.823	0.247
Jurkat	3,854.1	219.6	6%	3,299.2	303.9	9%	0.109	0.686

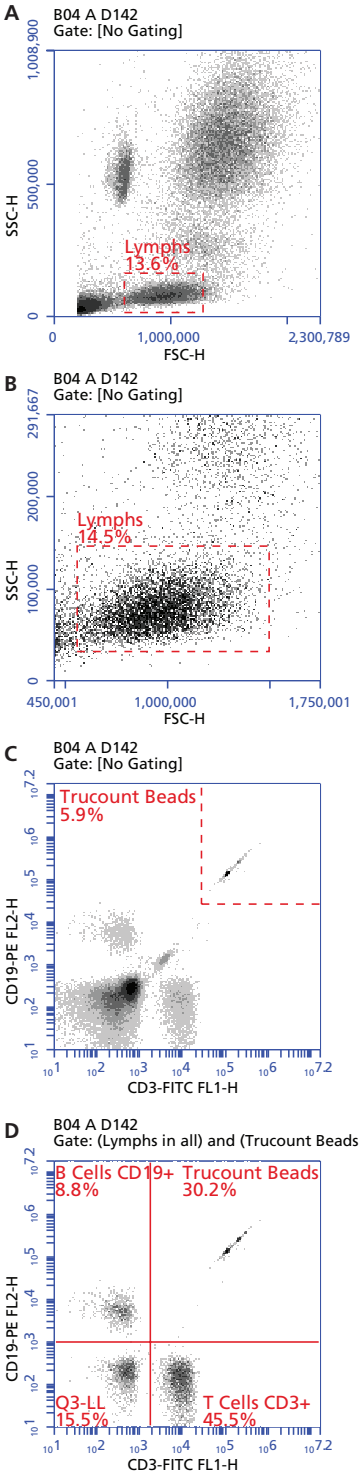


Figure 6. Identification of B- and T-cell subpopulations.

A. Lymphocytes were gated on an FSC vs SSC (side scatter) plot. B. The Zoom tool was used to adjust the gate for optimal selection of cells. C. Beads were gated on a fluorescence plot. D. B- and T-cell subpopulations were identified by CD19 and CD3 expression, respectively. Color compensation was applied to remove the effects of fluorescence spillover.

Figure 5 compares the counts obtained by the direct volume method for a dilution series of each culture. Each dilution series resulted in a linear correlation (R^2 > 0.999 for all cultures), suggesting that the cytometer was able to accurately report a broad range of concentrations.

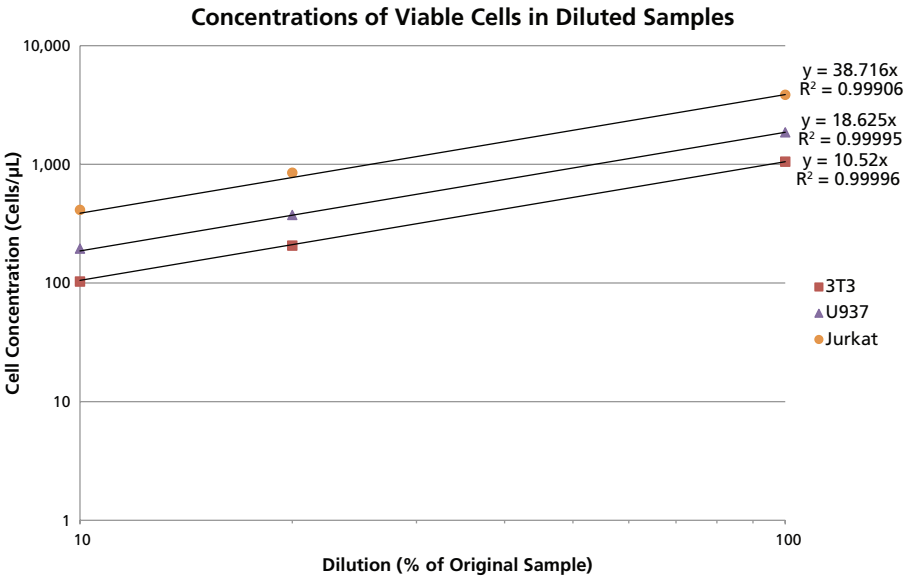


Figure 5. Serial dilutions of viable cells.

Comparison of viable cells per microliter determined by direct volume method using serial dilutions of 3T3, U937, and Jurkat cells. Axes are scaled logarithmically for easier comparison.

Immune cell counts

To determine B-cell (CD19⁺) and T-cell (CD3⁺) concentrations, human peripheral blood was collected from two healthy donors. Duplicate samples from each donor were prepared by adding 50 µL of blood to 5 µL each of CD3 FITC and CD19 PE antibodies and incubating for 20 minutes at room temperature in the dark. Red blood cells were lysed by adding 450 µL of BD FACS lysing solution according to the manufacturer's instructions. Cells were pelleted and resuspended in 500 µL of PBS + 5% bovine serum albumin (BSA), and 500 µL was transferred to BD Trucount tubes. Five thousand lymphocytes were collected from each sample on the BD Accuri C6 with an FSC-H threshold of 200,000.

When possible, it is best to minimize steps such as washes and buffer changes in order to minimize loss of sample during each pelleting and resuspension cycle. Proper titration of antibodies can reduce background fluorescence, alleviating the need for extra wash steps. In addition, whenever a different buffer is used, the operator must validate fluidics performance with reference beads in that sample buffer and calibrate the fluidics if necessary.

Thresholds should be determined empirically for each sample type to collect the complete population of interest while minimizing unnecessary data from debris or other cells.

After gates were set on lymphocytes (Figure 6A, B) and BD Trucount beads (Figure 6C), B cells (CD19⁺) and T cells (CD3⁺) were clearly distinguished on an FL1 vs FL2 plot (Figure 6D). *Gates should be set carefully in order to encompass the entire population of interest while excluding other cells. The Zoom function in BD Accuri C6 software can be helpful for setting the boundaries of the gates accurately (Figure 6B).*

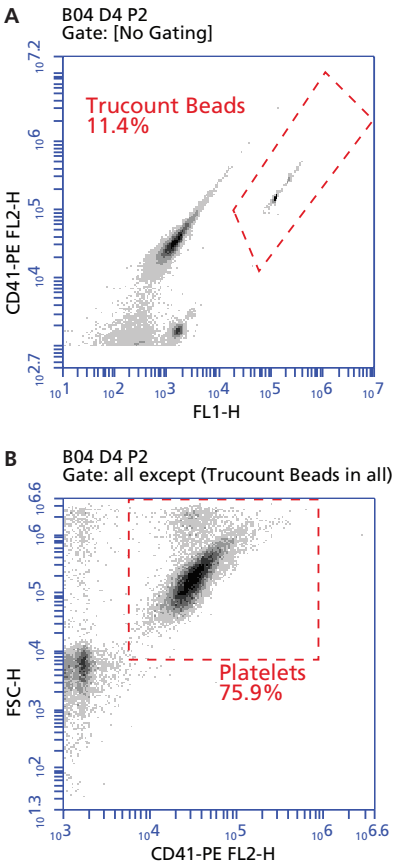


Figure 7. Platelet discrimination.

A. BD Trucount beads were identified and gated on an FL1 vs FL2 plot. B. With the beads excluded, the CD41⁺ platelets were identified and gated on an FL2 vs FSC plot.

In experiments using multiple fluorescent parameters, color compensation may be necessary to reduce the effect of fluorescence spillover from the primary detector into neighboring detectors. See the BD Accuri™ C6 Software User Guide for a more detailed discussion of fluorescence spillover and color compensation.

Table 2 shows immune cell statistics for two samples from two donors using the direct volume method. Results from duplicate samples were consistent, with %CVs less than 8%.

Table 2. B- and T-cell concentrations and %CV calculated using direct volume.

Donor	Sample	B-Cell (CD19⁺) Counts/µL (%CV)	T-Cell (CD3⁺) Counts/µL (%CV)
1	1	13.4 (6.9%)	83.7 (6.1%)
	2	16.8 (2.6%)	103.9 (7.5%)
14	1	25.2 (3.0%)	137.3 (6.5%)
	2	28.5 (4.0%)	149.0 (1.3%)

Platelet counts

Platelet concentrations were reported on the BD Accuri C6 in duplicate samples of human whole blood from two donors (method modified from Alugupalli, et al.[6]). Two-microliter aliquots of whole blood, collected in sodium citrate tubes, were diluted 1:10 into HEPES-buffered saline with 1% formaldehyde. Twenty-microliter aliquots of diluted blood were then incubated in 1.5-mL tubes for 20 minutes at room temperature with 20 µL of CD41 PE antibody. Samples were further diluted with 1 mL of HEPES-buffered saline with 1% formaldehyde, and 500 µL of diluted sample were aliquoted to BD Trucount tubes.

In addition to minimizing loss of sample, sample preparation should be adjusted to ensure optimal conditions for the samples, such as preventing clumping and enhancing their viability. For platelets, it is important to use a protocol that prevents their activation, which would lead to aggregation and impair accurate counting.

Samples were well mixed and acquired on the BD Accuri C6. Data was collected using an FL2-H (CD41 PE) threshold of 1,000. Dilution factors were applied to determine the platelets per microliter of the original whole blood sample.

The FL2-H acquisition threshold illustrates the delicate balance between excluding debris (and minimizing the amount of data collected) while still including the platelets and beads. Although the default FSC threshold of 80,000 works well for most cell types, it is too high for smaller cells such as platelets. Instead, the platelets' CD41 PE fluorescence in FL2 was used to set the threshold. After excluding the beads (Figure 7A), the platelets were clearly identified on an FL2 vs FSC plot (Figure 7B).

Table 3 shows platelet statistics for two samples from two donors using the direct volume method. As in Table 2, all %CVs are less than 8%. Adjusting for dilution yields platelet counts per microliter of blood.

Table 3. Platelet concentrations and %CV calculated using direct volume.

Donor	Sample	Platelet (CD41⁺) Counts/µL of Sample (%CV)	Dilution Factor	Platelet (CD41⁺) Counts/µL of Blood (%CV)
3	1	684.4 (4.8%)	520	355,867 (4.8%)
	2	719.4 (3.7%)	520	374,067 (3.7%)
4	1	607.6 (2.3%)	520	315,959 (2.3%)
	2	556.7 (7.1%)	520	289,487 (7.1%)

Direct volume vs counting beads

The ability of the BD Accuri C6 to determine volume directly eliminates the need to add counting beads to each sample. This can reduce costs significantly in experiments that analyze many samples.

Counting beads can also overlap with samples of interest. In Figure 3A, for example, the BD Trucount beads overlap Jurkat cells on an FSC vs FL3 plot. This overlap can interfere with accurate cell counts. The beads' broad fluorescence spectrum can help to resolve this issue. After identifying the bead population on an FL1 vs FL2 plot (Figure 3B), a gate was set to exclude them from the FSC vs FL3 plot (Figure 3D). Only then could the Jurkat cells be counted. In cases like this, where counting beads overlap with samples, the direct volume method is a convenient alternative that eliminates these complications.

Since all the experiments reported in this white paper used BD Trucount tubes, they were aggregated to compare the direct volume vs counting bead methods on the BD Accuri C6. A total of 48 different samples of human peripheral blood and mammalian cell lines were collected as described in earlier sections of this paper. All samples were collected in triplicate on the BD Accuri C6. As shown in Figure 8, the direct counts correlated highly (r^2 = 0.9989) with counting beads results.

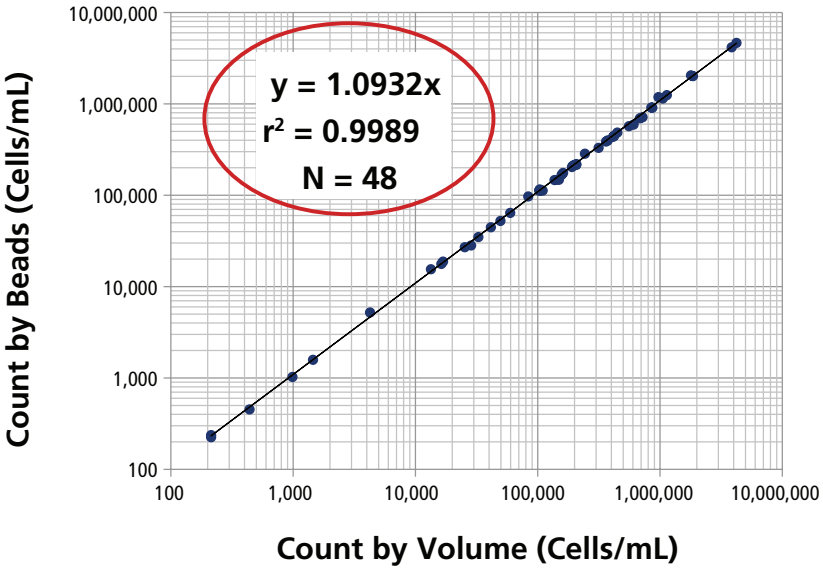


Figure 8. Comparison of cell concentration reported based on volume vs counting beads.

Serial dilutions of Jurkat, 3T3, and U937 cells, and T cells, B cells, and platelet samples from four human peripheral blood donors, were counted on the BD Accuri C6 by two methods. X-axis values represent volume reported, while y-axis values were calculated based on counting beads.

The high correlation validates the use of direct-volume reporting on the BD Accuri C6. Both the direct volume and bead counting methods are consistent across a wide range of concentrations. The slope of the line—about 1.09—indicates that the counting bead method consistently produces slightly higher concentration readings than the direct volume method.

Because both the direct volume and counting beads methods have their own intrinsic sources of error, this analysis cannot determine which is more accurate. For most experiments, in which cell concentrations are compared across different conditions, either technique, if used consistently, produces valid results over a

broad range of cell concentrations. However, counts by the two methods may differ. Therefore, if determining the absolute cell concentration is important, validating the chosen method with known standards is recommended.

Figure 9 and Table 4 illustrate a typical experiment comparing different conditions, in this case the concentration of dead cells for each cell line after culturing for one or two weeks. Dead cell counts increased significantly in the 2-week cultures of Jurkat and 3T3 cells ($p < 0.05$), but did not increase significantly in the U937 cultures.

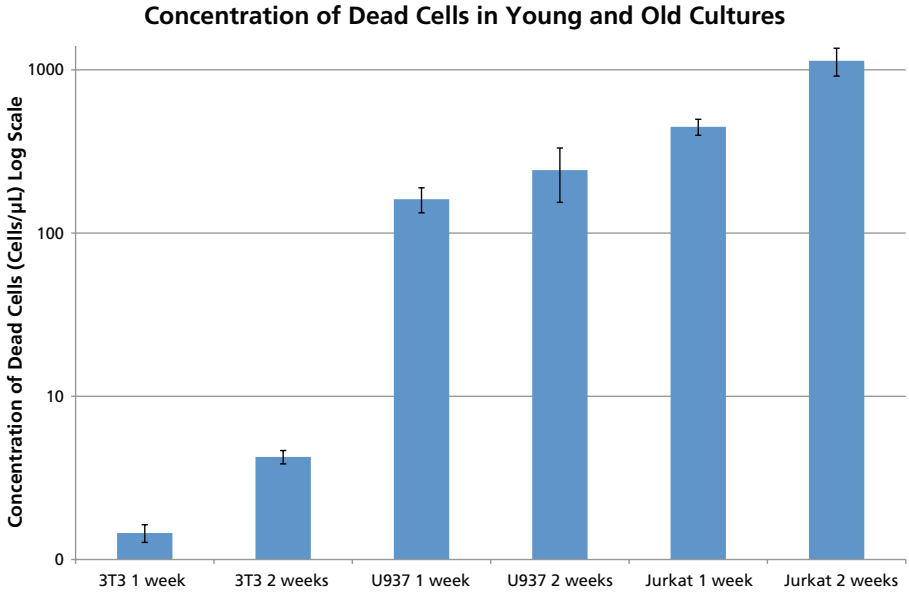


Figure 9. Concentration of dead cells in 1- and 2-week-old cultures.

Comparison of dead cells per microliter determined by the direct volume method in 1- vs 2-week-old cultures for three cell lines.

Table 4. Dead cell concentrations in three cell lines calculated using direct volume.

	1 week		2 weeks		
	Mean (cells/µL)	Std Dev	Mean (cells/µL)	Std Dev	Paired Student's t-Test*
3T3	1.5	0.2	4.3	0.4	**0.002**
U937	161.2	28.2	243.1	88.9	0.247
Jurkat	447.7	50.3	1,136.6	220.4	**0.027**

*T-tests indicate comparisons between 1- and 2-week means.

Discussion

Although hemocytometers are traditionally used to measure cell concentrations, their sources of error are well known,[7] and this study confirmed that they can be highly variable (the average %CV for replicate viable cell counts = 17.5%). In addition, hemocytometers cannot effectively count small particles such as platelets, or differentiate a subset of cells within a heterogeneous population.

Flow cytometers allow precise phenotypic identification of cell subpopulations using multiparametric sample analysis with single-cell resolution. By adding counting beads to a sample, standard laminar-fluidics flow cytometers can provide rapid and accurate cell concentration measurements as well. Counting beads save time and improve precision compared to the hemocytometer method,[8] and have largely replaced it when a flow cytometer is available. However, even this approach is subject to error sources such as pipetting technique and calibration, variability in bead-stock concentrations, and subjective bead gate settings. The beads are also an added expense.

The BD Accuri C6 flow cytometer features a unique peristaltic fluidics system that allows direct reporting of the volume sampled from cell suspensions. The system operates with standard laminar flow that enhances fluorescence and light scatter resolution. The current study demonstrated that volume reporting on the BD Accuri C6 offers a convenient method for determining concentrations of viable cells in cultured cell lines; immune cells in human peripheral blood; and platelets in whole, unlysed human peripheral blood.

Cell concentration determined by the volume method shares the advantages of counting beads, such as speed, accuracy, and analysis of complex populations, while minimizing their added cost and complexity. The counts correlate highly with counting beads and appear to be more precise than the hemocytometer method ($p < 0.01$ in one of three comparisons), making it easier to obtain significant results. The only data calculation required is to account for dilution factors, if applicable. It is not necessary to "back-calculate" the volume sampled based on the number of beads collected, or to decide subjectively how to gate on the singlet bead population.[9]

In sum, the BD Accuri C6 offers cell biologists an easy-to-use, affordable, and highly functional cell counter and flow cytometer, all in one.

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