Comparison of Count Reproducibility, Accuracy, and Time to Results between a Hemocytometer and the TC20™ Automated Cell Counter

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Tech Note

Cell Counting

Bulletin 6003

Introduction

For over 100 years the hemocytometer has been used by cell biologists to quantitate cells. It was first developed for the quantitation of blood cells but became a popular and effective tool for counting a variety of cell types, particles, and even small organisms. Currently, hemocytometers, armed with improved Neubauer grids, are a mainstay of cell biology labs.

Despite its longevity and versatility, hemocytometer counting suffers from a variety of shortcomings. These shortcomings include, but are not limited to, a lack of statistical robustness at low sample concentration, poor counts due to device misuse, and subjectivity of counts among users, in addition to a time-consuming and tedious operation. In recent years automated cell counting has become an attractive alternative to manual hemocytometer–based cell counting, offering more reliable results in a fraction of the time needed for manual counting.

This report compares the precision of cell counts obtained with a hemocytometer to those obtained by automated cell counting using Bio-Rad's TC20 automated cell counter. Sources of error that are inherent to the device, and those introduced by the operator, are investigated. We demonstrate that automated cell counting can significantly reduce user- and concentration-dependent count variance, while greatly reducing the time needed to perform counts.

Methods

Cell Culture

HeLa cells were grown in advanced DMEM containing 1x sodium pyruvate and nonessential amino acids (Life Technologies Corporation) supplemented with 10% fetal bovine serum (Thermo Scientific). Detachment from plates was performed using enzymatic digestion of surface proteins by trypsin (Life Technologies Corporation) followed by neutralization with two volumes of growth medium. Jurkat cells were grown in RPMI medium containing 10% fetal bovine serum.

Beads

Polystyrene beads, 10 µm, were purchased from Life Technologies Corporation. Bead dilution was performed by adding beads to 1x DPBS (Life Technologies Corporation). Bead counts were performed by sequentially loading and counting the same chamber of a Bright-Line glass hemocytometer (Hausser Scientific). This was repeated ten times. The number of beads was recorded for all nine 1 x 1 mm grids.

Flow Cytometry

Flow cytometry was performed using a BD FACSCalibur flow cytometer (BD Biosciences) and CountBright counting beads (Life Technologies Corporation). Medium containing 50,000 CountBright beads was combined one-to-one with 250 µl of cells in suspension, yielding a final solution containing 100 beads/µl. This solution was run through the flow cytometer until 10,000 events were collected in the gate previously defined as appropriate for non-doublet beads in the FSC x SSC channel.

Manual Counting

A preloaded plastic hemocytometer (INCYTO Co., Ltd.) was loaded with HeLa cells and the openings were sealed with tape to prevent evaporation. Individual counters were asked to count all cells within the 9 x 9 mm Neubauer grid. All counters used the same microscope and 10x objective. This was performed using two chambers and seven counters, yielding 14 total counts for each concentration. Time to count was measured from the moment the counters started looking through the eyepieces to when they reported their counts.

Automated Counting

Jurkat cells were counted by loading into a TC20 automated cell counter using the capillary-filled disposable loading chambers. Data were collected from four replicates on six distinct TC20 cell counters.

Prediction of the Coefficient of Variation (CV) for Defined Hemocytometer Area

Calculation of expected variation due to stochastic distribution of 10 µm beads was made using the following formula: (square root of expected/expected) x 100. The "expected" bead concentration is based on the number of beads that would be present in a perfectly formed and filled hemocytometer, given a defined area and concentration.

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Results and Discussion

Hemocytometer Count Variance Based on Area and Cell Concentration

An experienced user counted 10 µm beads loaded into a single chamber of a glass hemocytometer. All nine 1 x 1 mm areas were individually counted in the order illustrated in Figure 1. This operation was repeated for ten separate chamber loads. The concentration-dependent variation determined by experimentation is presented in Table 1. The same data were also plotted against the theoretical CV values based on a perfectly formed and filled hemocytometer (Figure 2).

A theoretical vs. experimental comparison was performed to demonstrate the CV limitations when using a hemocytometer. The data clearly demonstrate an increase in counting variation that is both area dependent and concentration dependent. The theoretical CV trend is matched closely by the experimental measurements. Both sets of data demonstrate an exponential increase in CV between 4 x 10^5 and 5 x 10^4 beads/ml. The inflection point for the transition between linear and exponential CV increase is area dependent as illustrated by the improved CVs when larger areas are analyzed. This shows that an experienced user can count beads with a precision close to the theoretical limit.

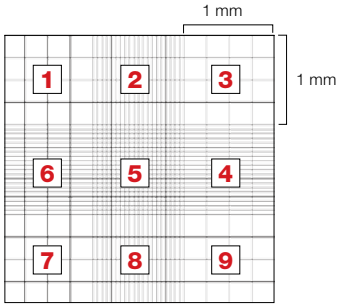

Fig. 1. Neubauer counting grid. The grid is divided in nine 1 mm² sections. Numbers indicate the order in which the sections were counted.

Table 1. Analysis of cell count variance at different cell concentrations and counting surface areas.

Flow Cytometry Concentration, beads/ml	Regions Analyzed	Total Surface Analyzed, mm²	Average Count/ mm²	SD	CV, %	Hemocytometer Concentration, beads/ml
9.3 x 10^6	5	1	112.4	12.0	10.7	1.1 x 10^6
9.3 x 10^6	1, 3, 7, 9	4	114.6	5.4	4.7	1.1 x 10^6
9.3 x 10^6	1–9	9	113.5	4.7	4.1	1.1 x 10^6
4.4 x 10^5	5	1	48.3	7.9	16.4	4.8 x 10^5
4.4 x 10^5	1, 3, 7, 9	4	45.7	4.5	9.8	4.6 x 10^5
4.4 x 10^5	1–9	9	45.9	3.0	6.5	4.6 x 10^5
5.1 x 10^4	5	1	6.2	2.1	33.8	6.2 x 10^4
5.1 x 10^4	1, 3, 7, 9	4	5.4	1.3	23.6	5.4 x 10^4
5.1 x 10^4	1–9	9	5.6	1.3	23.4	5.6 x 10^4

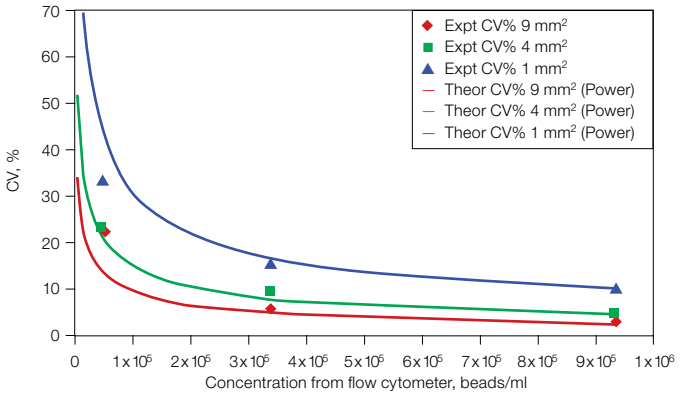

Fig. 2. Calculated theoretical CV values (lines) compared to experimental data (shapes). The calculated theoretical CV as it relates to concentration and area is derived by the following formula: (square root of expected/expected) x 100. The "expected" bead concentration is based on the number of beads that would be present in a perfectly formed and filled hemocytometer, given a defined area and concentration. The line fit to the derived values was performed using the nonlinear line-fitting method (Power) available in the Microsoft Excel graphing features. Expt, experimental; theor, theoretical.

While all counting methods are subject to variation, the hemocytometer is particularly sensitive at lower concentrations. Hemocytometer load-to-load CVs less than 10% are not likely at concentrations lower than 1 x 10^5 cells/ml and are area dependent up to 4.5 x 10^5 cells/ml. The transition point from linear to exponential increases of CV values varies with the area counted per load. To gather accurate data from a hemocytometer, the particle per cell concentration should be used to dictate the counting area to use for each load.

Hemocytometer Counting Error between Users

The data demonstrate theoretical limits of the hemocytometer. However, there are additional limitations that hamper the accuracy of hemocytometer counts. Paramount among these limitations is variation among users. When counting a cell population with a hemocytometer, users are faced with a variety of error-inducing situations. These situations include cells that lie on the grid lines, debris, clusters, and cell tracking. The data presented in Figures 3A and 3B demonstrate this inherent source of error. A HeLa cell population was prepared in complete growth media and loaded into a plastic hemocytometer (INCYTO). Once loaded, the openings were sealed to prevent evaporation. Seven experienced hemocytometer users were asked to count all cells within the 3 x 3 mm grid. The counters were not given instructions about what to do with cells on the lines or in clusters or debris. The only instructions were to count cells within the entire 3 x 3 grid. The sample provided was well distributed with relatively few clusters or debris. HeLa cells are roughly 15–20 µm, allowing them to be easily identified using a phase contrast 10x objective. The microscope was preset to this objective. A sample image taken on a TC20 automated cell counter (Figure 4) demonstrates the nature of the samples used for hemocytometer counting.

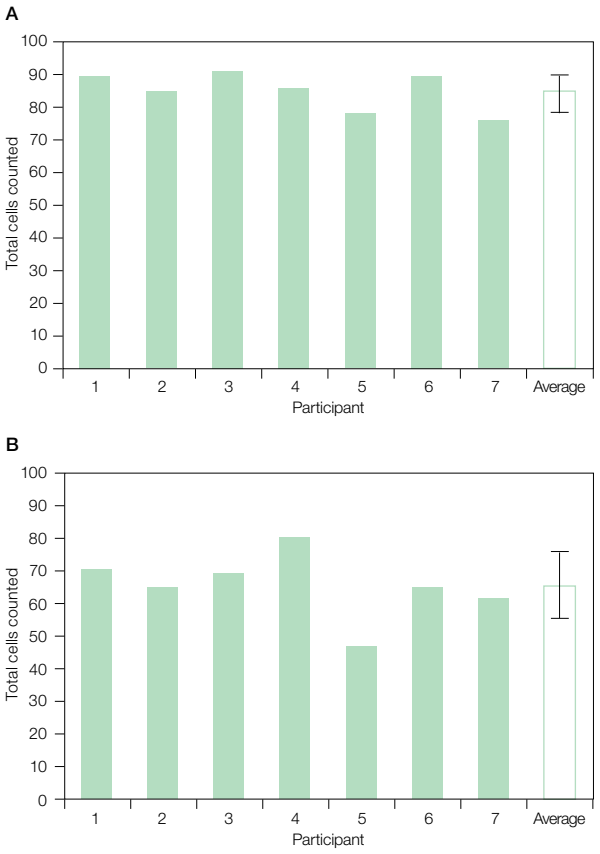

Fig. 3. Analysis of user-based variance in manual counts. Seven individuals were given two HeLa cell samples with a concentration of 1 x 10^6 cells/ml (**A**) and 4 x 10^5 cells/ml (**B**). Total cell counts of the two samples were reported for each individual. CVs of 7.1% and 15.6% were calculated for the low and high cell concentration samples, respectively.

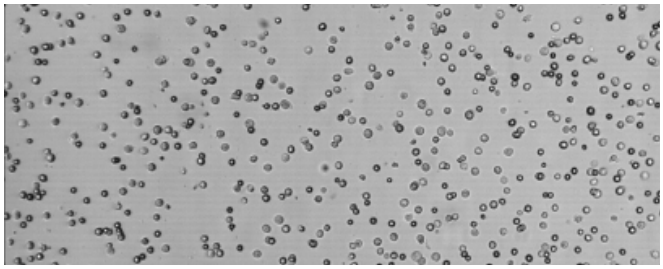

Fig. 4. Image of a HeLa cell sample used for the user-based variance experiment. This image demonstrates the relatively uncomplicated nature, without cell clusters, of the sample counted.

Multiple counts of the same sample by different users revealed the inherently imprecise nature of hemocytometer counts from operator to operator. The CV between hemocytometer users (Table 2) ranged from as low as 7.1% to as high as 15.6%. Figures 3A and 3B are presented as the best (1 x 10^6 cells/ml) and worst (4 x 10^5 cells/ml) case examples from the data set, respectively. Training may allow for individual laboratories to normalize counts among users, but many of the differences are due to the subjective nature of cell determination, cluster disaggregation, or debris rejection. The skills required to carry out these activities are generally honed through years of practice and are therefore difficult to teach.

Automated Cell Counting Using the TC20 Automated Cell Counter

The use of automated devices, such as the TC20 cell counter, can eliminate much of the subjectivity by applying algorithms trained to identify cells, disaggregate clusters, and effectively reject debris. To investigate the potential advantage of automatic cell counting, the following experiment was conducted to assess multi-instrument counting of the same sample (Figures 5A–D; Table 3): one chamber was loaded with Jurkat cells at 1 x 10^6 cells/ml, and the chamber was measured using six separate TC20 cell counters. This procedure was repeated four times. The largest CV for this set of experiments was 2.4% (Table 3). The CV, when comparing human counters in the previous experiment, was as large as 15.6% (Table 2). The increased precision of the TC20 cell counter is achieved by replacing human subjectivity with objective choices embedded in an algorithm.

Sources of error using a hemocytometer are well understood, and are often avoided in the hands of a skilled user. However, the time required to count cells, the tedious nature of the procedure, and the strain on the user are endemic to the device. Counting using a hemocytometer generally requires a phase contrast microscope, the hemocytometer itself, and a tracking device, such as a handheld or tabletop manual counter. This setup can cost from a few hundred dollars to several thousand dollars. More importantly, the operation of a hemocytometer requires proper washing, handling, and loading of the device. Failure to do so can introduce additional sources of error not addressed in this report. Once ready to count, the operator will have to perform multiple focusing, repositioning, and counting steps to collect the final count. This can impose a significant

Table 2. Analysis of cell count variance between individuals at different cell concentrations.

Replicate	Flow Cytometry–Derived Concentration, cells/ml	Individual Hemocytometer Cell Counts* 1	2	3	4	5	6	7	Average	SD	CV, %	Hemocytometer-Derived Concentration, cells/ml
1	7.4 x 10^5	804	760	819	775	700	801	678	762.4	54.2	7.1	8.4 x 10^5
	3.9 x 10^5	296	318	298	328	225	319	260	292.0	37.1	12.7	3.2 x 10^5
	1.7 x 10^4	29	29	30	31	21	25	23	26.9	3.8	14.3	2.9 x 10^4
2	7.4 x 10^5	834	607	808	830	673	733	722	743.9	85.6	11.5	8.3 x 10^5
	3.9 x 10^5	369	322	335	344	251	336	309	323.7	37.1	11.5	3.6 x 10^5
	1.7 x 10^4	38	35	37	43	25	35	33	35.1	5.5	15.6	3.9 x 10^4

* Numbers in red are values that deviate from the average cell count by more than 5%.

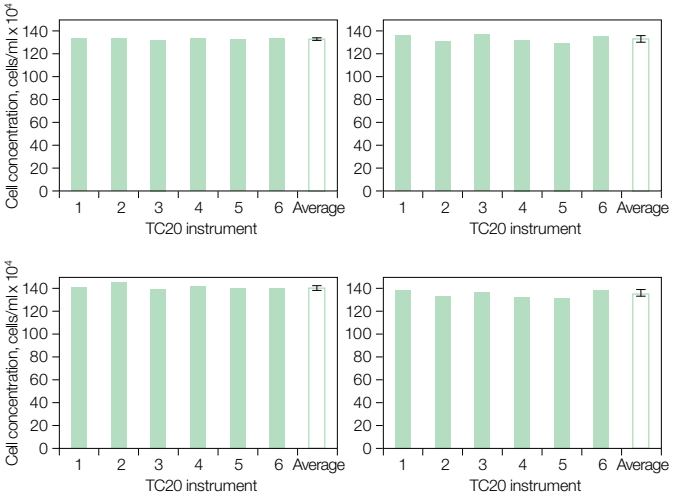

Fig. 5. Analysis of automated cell count reproducibility. Four samples of Jurkat cells at a concentration of 1 x 10^6 cells/ml were counted on six different TC20 automated cell counters. Green bars represent the cell count obtained for each individual TC20 instrument. White bars represent the average cell count of all six instruments. Error bars = 1 SD.

Table 3. Analysis of cell count variance between TC20 instruments.

	TC20 Instrument						Average Cell		
Replicate	1	2	3	4	5	6	Count x 10^6	SD	CV, %
1	134.0	134.0	132.0	134.0	132.0	134.0	133.3	1.0	0.8
2	136.0	131.0	137.0	132.0	129.0	135.0	133.3	3.1	2.4
3	139.0	144.0	139.0	140.0	138.0	139.0	139.8	2.1	1.5
4	138.0	133.0	136.0	132.0	132.0	137.0	134.7	2.7	2.0

time burden on the research. The times to count data were concurrently collected for the set of experiments described in Table 2 and Figure 3 and are presented in Figure 6, which displays a nearly linear relationship between cell concentration and time to count. At the lowest concentration, the count required an average of 26 to 33 seconds for replicates 1 and 2, respectively. The majority of this time was spent repositioning the slide and refocusing. At the highest concentration, the average time to count averaged 292 and 308 seconds (roughly 3 minutes) for replicates 1 and 2, respectively. In this case, the majority of time was spent actually counting cells. Both low concentration, ~4 x 10^4 cells/ml, and high concentration, ~8 x 10^5 cells/ml, required more time to count manually compared to counts performed with the TC20 cell counter, which required only ~20–30 seconds.

These data demonstrate two critical deficiencies in cell counting with the hemocytometer — count variation among users and time to count. Multiple users counting the same chamber resulted in CVs as large as 15.6% (Table 2), while the same sample counted by multiple TC20 instruments resulted in CVs not exceeding 2.4% (Table 3). Time to count with a hemocytometer is highly concentration dependent (Figure 6). At the same concentrations the TC20 cell counter required less than 30 seconds, regardless of concentration.

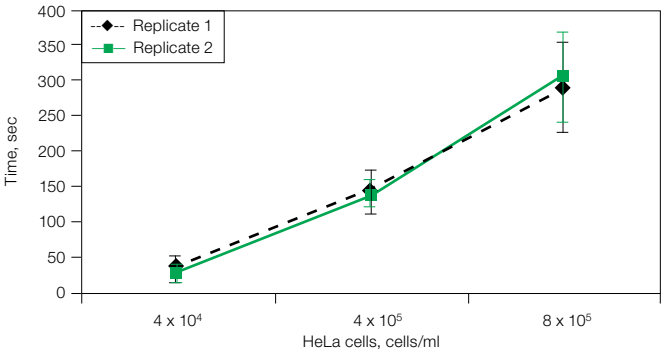

Fig. 6. Time to results required to count different concentrations of HeLa cells using a hemocytometer. Error bars = 1 SD; n = 7.

Conclusions

The rapid time to count, removal of human subjectivity, and iterative improvements to counting algorithms offered by an automated cell counter, such as the TC20 cell counter, make it preferable to manual hemocytometer counting. A hemocytometer in the hands of an expert user will continue to be a capable device. However, in the era of high-throughput and multidisciplinary science, automated counting will become a necessity in research laboratories

For more information, visit
www.bio-rad.com/web/TC20vsHemo.

Information in this tech note was current as of the date of writing (2010) and not necessarily the date this version (rev B, 2013) was published.

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