BECKMAN COULTER
Life Sciences

Beadless Absolute Counting

Application of the unique properties of the peristaltic pump fluidic based system for volumetric cell counting

Pierre Grenot, C.Cy (ISAC), Hervé Luche, Ph.D., Centre d'Immunophénomique - CIPHE (PHENOMIN), Aix Marseille Université (UMS3367), Inserm (US012), CNRS (UMS3367), Marseille, France.

Introduction

The enumeration of particles in solution is of interest across a broad range of biological fields including basic and clinical research as well as industrial and environmental microbiology. Traditionally, manual or semi-automated methods using counting chambers or slides are used for this purpose. However there is a high degree of variability in this method attributed to operator error.[1]

Due to the ability to resolve single particles of different sizes, flow cytometry is particularly suited to cell counting. Common methods rely on the use of external calibrator beads of known concentration that are spiked into the sample. By calculating a ratio of the number of beads observed on the cytometer to the expected number of beads spiked into the sample, it is possible to calculate the concentration of other particles of interest. However, as beads tend to stick to plastic tubing, this counting method has a tendency to over-estimate the sample concentration. In addition, counting beads are expensive and substantially increase the cost of the assay.

Objectives

- Compare and contrast syringe pump and peristaltic pump fluidics
- Learn how to monitor peristaltic pump fluidic stability to ensure accurate absolute counting
- See a validation protocol for establishing an absolute counting assay for mouse splenocytes

Flow cytometers using volumetric syringes for sample introduction remove the need for calibrator beads as particles are counted in a set volume injected in the system. Once injected in the system, the sample is pushed to the flow cell by sheath fluid. Usually the maximum injection volume is limited to 500 µL. Therefore, in using volumetric syringes for absolute counts it is necessary to check the stability of events counted over time to ensure there is no dilution effect at the end of the acquisition. Counting time frame should be adjusted on a constant time interval. In contrast, the peristaltic pump system of CytoFLEX LX allows one to record an unlimited sample volume (up to 4 mL), without any dilution effect.

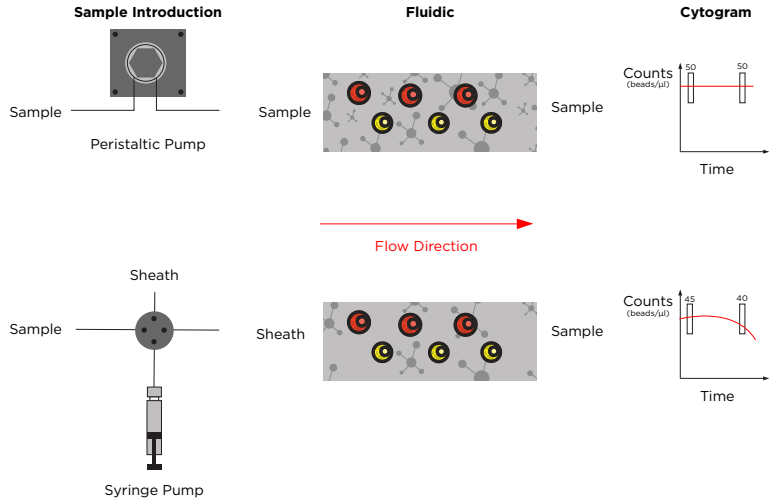


Figure 1. Comparison of Syringe and Peristaltic Pump on Cell Counting Measurements. In the syringe pump system a dilution effect can be observed due to sheath injection at the beginning and end of the sample injection. The peristaltic pump provides continuous and stable acquisition throughout the entire sample volume. Stability is checked with gates at the beginning and end of acquisition by plot concentration over time.

Fluidic System Performance Monitoring

As described in the introduction, the peristaltic pump is capable of stable and consistent sample introduction. This is achievable as long as the overall system fluidics system is in proper working condition. When relying on volumetric counting assays in your laboratory it is important to first establish routine procedures to assess fluidic stability over time so that issues can be identified and addressed with routine maintenance procedures.

Here we demonstrate a bead based quality assurance test to verify counting performance. A specific lot of counting beads is run on the instrument prior to completing the counting experiment. Using a Levey-Jennings plot, figure 2, changes in performance over time can be observed. If the change falls outside of the established range the operator should implement system cleaning procedures. If that doesn't resolve the issue then performing the routine maintenance procedures to replace the sample injection or system tubing may be necessary.

Although the quality assurance test requires the use of beads, the overall volume is much less than adding beads to each sample as is done in bead based counting assays.

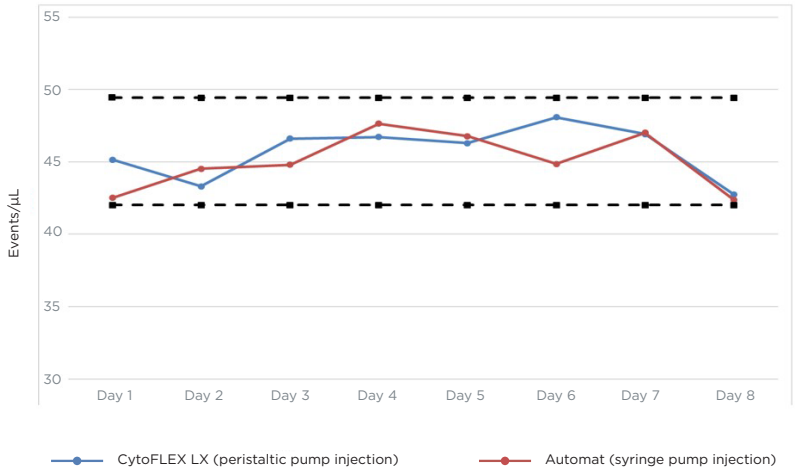
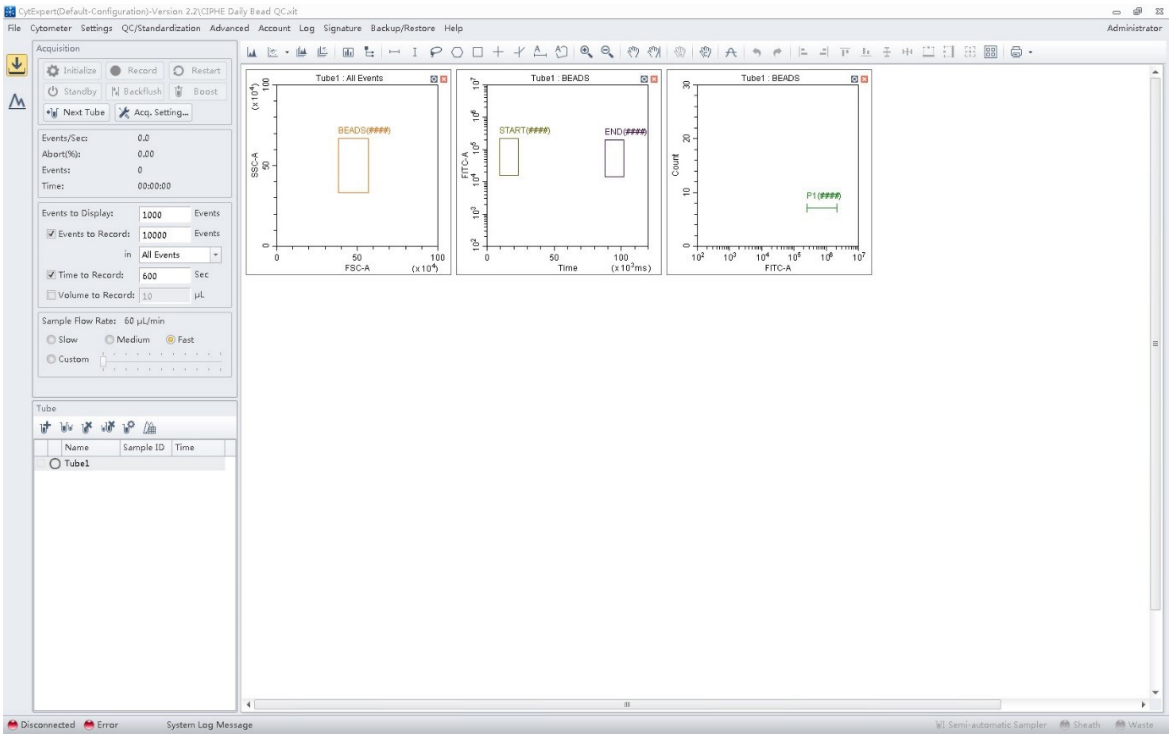


Figure 2. Quality Assurance Monitoring. Levey Jennings plot of known lot of counting beads measured over time to detect changes in the fluidic system that could compromise the counting assay. The control range is established during assay development, mean +/- 2 times the standard deviation (black lines). Daily measurements are collected and added to the chart to ensure performance is within the established ranges. This graph includes the measurements taken from an established assay using the syringe pump system (red line) and the comparison measurements taken with the CytoFLEX, a peristaltic pump containing system (blue line). Since the measurements with the two different systems tend to correlate with each other, we can deduce that some of the assay variation is introduced by the counting beads.

Quality Assurance Procedure

1. Run the Daily QC-including Daily QC and Daily IR QC Fluorospheres- according to the manufacturer's Instructionsfor Use (PN B49006xx). Refer to the section Instrument Quality Control and Standardization.
2. Exit the QC/Standardization screen.
3. Perform Daily Clean- refer to procedure in the section entitled Cleaning Procedures- following the prompts as they appear on the screen.
4. Select file-New Experiment. Create the following plots as presented below:
5. Record the counting beads for 10,000 total events at 60 µL/min. To run daily you may either:
   - A Add a tube to the existing experiment
   - B Save the experiment as a template that can then be recalled daily or as needed
6. Repeat step 3.
7. Create the Levey-Jennings plot: open the stats table, batch export the CSV file and update daily.
8. Compare the values given by the manufacturer for the lot of counting beads and to the ranges in the Levey-Jennings plot.



Counting Assay Optimization

The power of flow cytometry comes from its ability to analyze multiple physical characteristics simultaneously on a large numbers of events in a heterogeneous sample. Using these measurements specific subpopulations can be identified. Adding the cell counting method to the assay means that each identified sub population can now be enumerated, unlike manual cell counting methods.

Here we apply this technique to a sample of mouse splenocytes, distinguishing the white and red blood cells in the sample. The first step in developing this assay is to optimize the population separation detected by the flow cytometer. This is achieved by calculating the Separation Index[2] for these populations as the gain for the Forward and Side Scatter detectors are adjusted. Figure 3 is a graph of the Separation Index by FSC gain setting and figure 4 provides representative plots from the study.

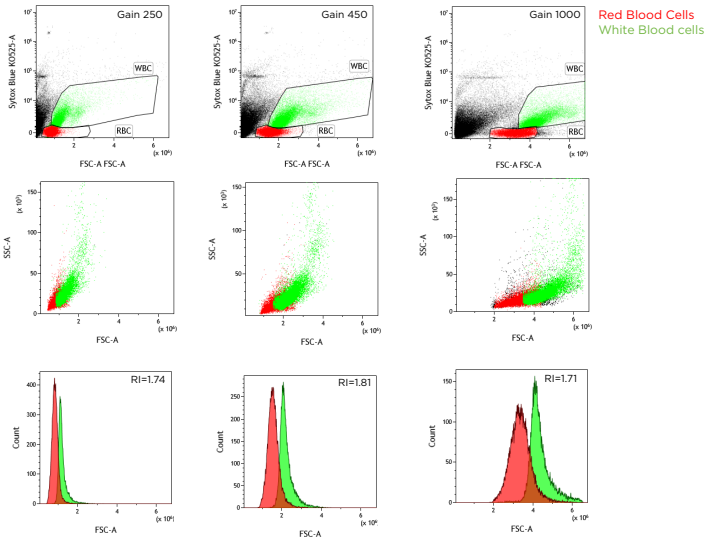


Figure 3. Resolving RBC and WBC in Whole Spleen Extract. Spleen extracts without erythrocyte lysis were stained with SYTOX™ Blue dead cell stain, detected in the KO525 channel. Using this plot, the gates for RBC and WBC were drawn based upon Forward Scatter profiles, row 1. Row 2 shows the same samples in a FSC x SSC plot, red is the RBC population and green is the WBC population. Overlay histograms based upon FSC are provided in row 3. These analyses were completed at various Forward Side Scatter gain settings. Representative plots at 250, 450, and 1000 gain setting (column 1, 2 and 3, respectively) are shown demonstrating the effect on visualizing the population separation.

Panel A

$$\text{Resolution Index} = \frac{\text{Median pos - Median neg}}{\sqrt{\text{SDpos}^2 + \text{SDneg}^2}}$$

Panel B

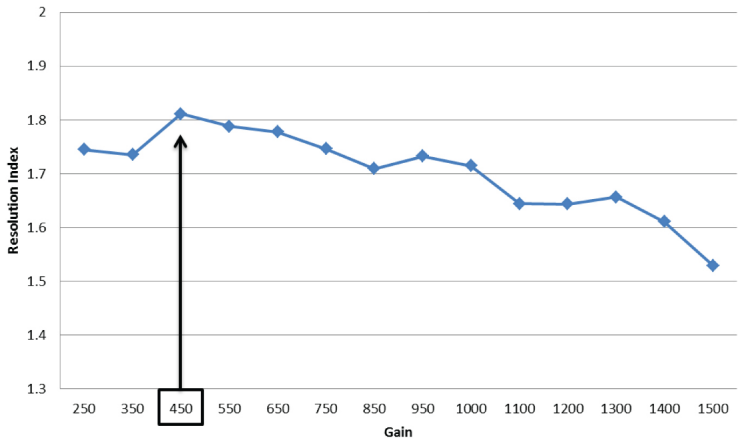


Figure 4. Determining Optimal FSC Gain Setting. The spleen samples were analyzed at various FSC gain settings, from 150 to 1000 in increments of 100. The Resolution Index for RBC and WBC populations (see gating in figure 4) were calculated using the equation and graphed versus the gain setting (Panel A). The maximal resolution of WBC from RBCs was obtained at gain setting of 450 (Panel B).

Additional parameters can be added to the assay to increase the robustness of the population identification. We demonstrate an immunophenotyping approach to further define RBC and WBC populations. This method relies on fluorescent staining using markers for these cell types. CD45 is ubiquitously expressed on the white blood cell, lymphocytes. Ter-119 is a marker for the red blood cell, from early proerythroblast to mature erythrocyte stages.

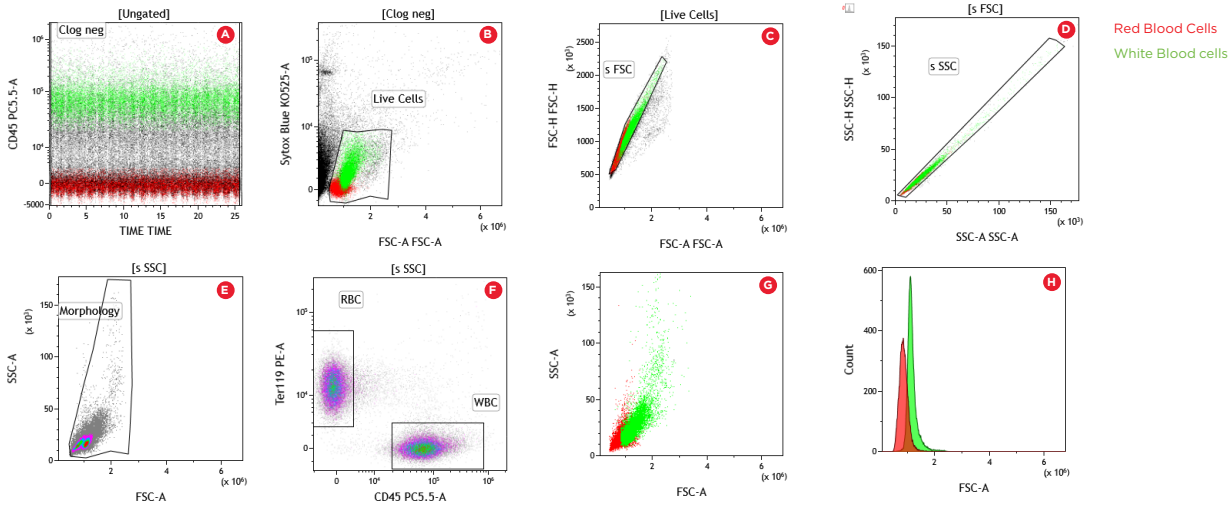


Figure 5. Gating Strategy and Acquisition Controls for Splenocyte Counting Assay. To ensure that system fluidics are stable, monitor the acquisition with a time plot, plot A. SYTOX™ Blue dead cell stain, detected in the KO525 channel is used to gate on live cells, plot B. Dead cells can bind to labeled antibodies in a nonspecific manner, increasing the background. Plots C and D show two doublet discrimination methods, based on forward scatter and side scatter respectively. The FSC x SSC plot shows the scatter profile of the live, singlet events, plot D. The morphology gate was used for the remaining analysis, plot E. The CD45 x Ter119 plot is used to identify the WBC and RBC populations, plot F. The scatter profile for these populations is shown in plot G demonstrating that WBC and RBC have different scatter profiles and the histograms for these two populations are shown in an overlay, plot H. The addition of population specific markers yielded a more robust delineation of WBC and RBCs.

Counting Assay Validation

In the introduction we discussed the unique properties of the peristaltic pump in regards to the sample introduction method. Calibrating the flow rate prior to acquisition is vital, otherwise results may be inaccurate. When the detection rate of a sample is less than or equal to 10,000 events/second, the sample flow rate should be between medium and high i.e., 30 µL/min to 60 µL/min to ensure accuracy. Samples with higher event rates must be acquired using the High event rate setting; this adjusts and optimizes the system's ability to acquire events with a lower abort rate.

Theoretically, the sample should not experience any dilution effects as it is injected into the fluidic stream as sheath fluid is not used to boost the flow. Assessing the sample concentration at the beginning and end of the run allows for an assessment of this robustness of this phenomenon, as depicted in figure 6. This plot is also indicative of when and how often the tubing should be changed. The steadiness of the sample flow indicates intact sample tubing; as the tubing becomes worn and needs to be replaced undulating effects are observed.

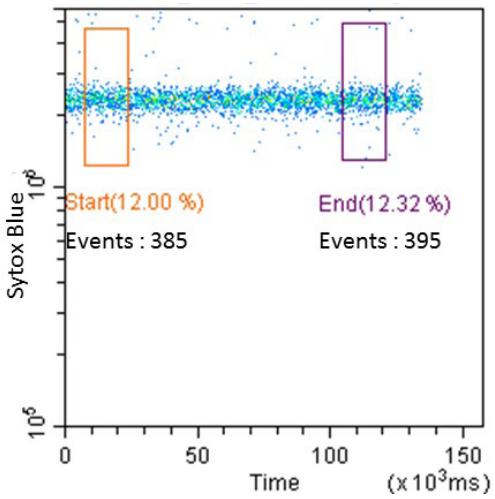


Figure 6. Stability of sample cell counting on the CytoFLEX LX. The time parameter was used to assess sample dilution. Two cell counts were determined using time segments at the beginning and the end of sample acquisition. Consistent values, 385 events at the beginning versus 395 at the end of the run, indicate stable acquisition achieved by the peristaltic pump sample introduction.

Another important instrument property to consider in relying on counting data is the sample carryover, or the events attributed to the previously analyzed sample being observed in subsequent sample runs. This instrument specification is critical in absolute counting applications. We devised as simple method to assess cross contamination in the cell counting assay, see figure 7. Using this method we generated data and assessed cross contamination between the two sample types, see figure 8.

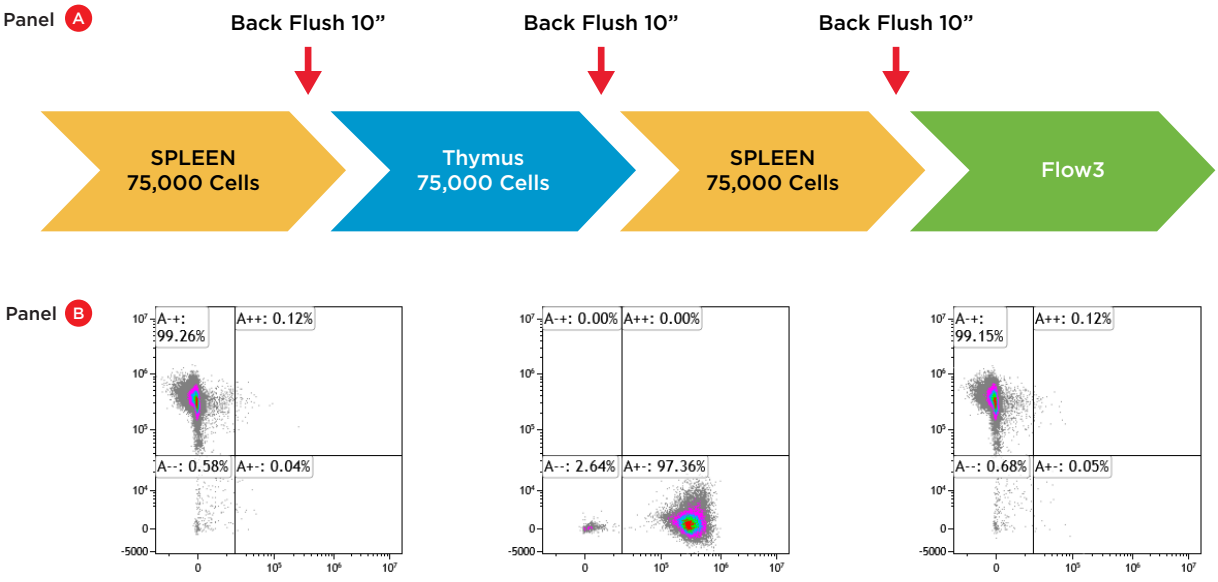


Figure 7. No Cross-contamination Observed in Sequential Samples. Spleen cells and thymus cells were both stained with CD45, but with different fluorochromes. Samples were analyzed sequentially with a 10 second backflush between samples (Panel A). Spleen cells were stained with CD45-APC-A700 and Thymus cells were stained with CD45-BV510. Samples were acquired sequentially on the CytoFLEX LX flow cytometer. No APC-A700 signal was detected in the thymus sample. No BV510 signal was detected in the second spleen sample (Panel B).

The absolute counting assay was compared to the reference method using counting beads. Samples were spiked with a known concentration of counting beads and the resulting observed counts were compared, see figure 8. The two methods showed a strong correlation.

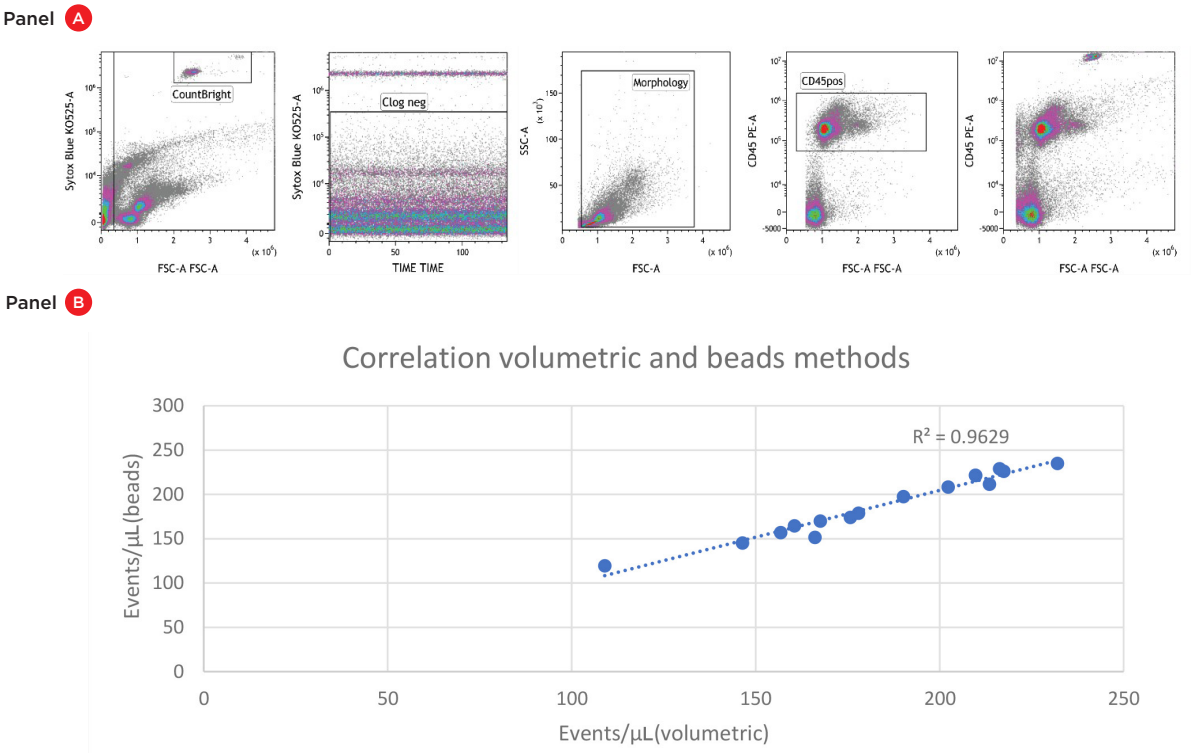


Figure 8. Assay Verification Using Spiked Counting Beads. Mouse spleen cell preparation was stained and spiked with a known quantity of CountBright™ absolute counting beads prior to acquisition. Gating and acquisition controls were assessed as described (see figure 5). Events from the "CountBright" and "CD45pos" gates were counted (Panel A). Cell counts from absolute counting method were plotted versus the calculated results from the beads. A strong correlation between the two values was obtained (Panel B).

Another verification study was completed comparing syringe based and peristaltic pump based flow cytometers. Similar degrees of variation were observed among the samples analyzed and a strong correlation across methods was observed.

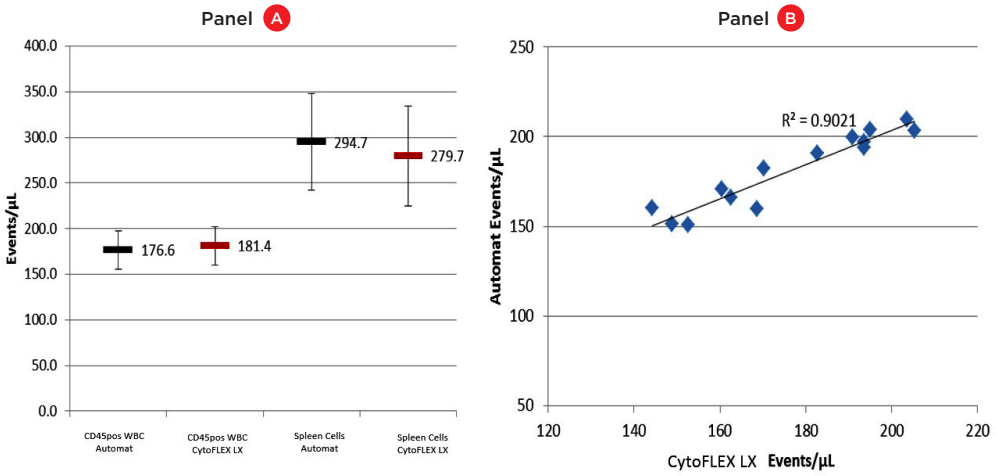


Figure 9. Assay Comparison on Two Different Platforms. Comparison of results between syringe based (Automat) and peristaltic pump based (CytoFLEX LX) over the entire cohort study (n=15). In panel A, the mean and standard deviation from total cells and gated WBCs is graphed. Results indicate a similar mean and variability across both methods. In panel B, scatter plot of the results from each sample on each instrument are graphed demonstrating strong correlation between the two values was obtained, r^2=0.90.

Conclusion

In this paper we describe an absolute cell counting method using a flow cytometric approach that is not based upon the addition of an internal counting fluorosphere. This allows investigators to harness the power of multiparametric analysis to identify cell populations of interest but moreover to enumerate them in the same assay. The ability to obtain accurate cell counts without the use of expensive beads can streamline the procedure.

The method developed in this paper utilized the CytoFLEX LX flow cytometer. This instrument uses a peristaltic pump based fluidics system. Accurate enumeration is achievable as long as the fluidics system is maintained in good working condition. An efficient quality assurance method has been provided that allows investigators to monitor performance and address with routine maintenance procedures as needed.

The CytoFLEX flow cytometer with its peristaltic pump sample injection has some advantages over syringe based systems in the area of absolute counting. These include the ability to count from much larger sample volumes and stable cell counts over time. In the assay verification studies, we demonstrated robust performance and very low sample carryover.

References:

1. Freund M, Carol B. Factors Affecting Haemocytometer Counts of Sperm Concentration in Human Semen. Journal of Reproductive Fertility 1964; 8: 149-55.
2. Telford, W. G., Babin, S. A., Khorev, S. V. and Rowe, S. H. (2009), Green fiber lasers: An alternative to traditional DPSS green lasers for flow cytometry. Cytometry, 75A: 1031-1039. doi:10.1002/cyto.a.20790

FLOW-3741WP05.18