Poster #140

Improved Reproducibility and Repeatability in Full Spectrum Absolute Cell Counting

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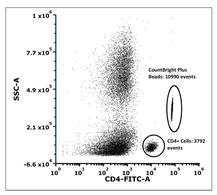
Abstract

Recent advances in flow cytometry have allowed for the study of cells in greater detail using highly multiplexed flow cytometry panels, allowing researchers to identify and better understand unique cell populations. Despite continued advancements in detection technologies, most flow cytometers are not equipped to provide absolute cell counts for identified cell populations of interest. Cell counts can be critical for many research, diagnostic and quality applications. To achieve absolute cell counting, an internal reference material of known concentration and volume can be used. These reference standards are typically 3–6-micron sized particles that have been internally stained or surface reacted with fluorescent dyes. Supplied as a suspension, the particles must be carefully pipetted into a sample of known volume before running on a flow cytometer. The cell-to-bead ratio then is used to calculate the absolute cell counts. Invitrogen™ CountBright™ Plus Absolute Counting Beads, recently introduced by Thermo Fisher Scientific, provide significant advantages over existing beads for cell counting including full spectral excitation and emission as well as forward and side scatter properties well suited to cellular analysis. However, it has been found that the accuracy and reproducibility of cell counting using absolute counting beads can be impacted by instrument maintenance, pipetting errors, bead aggregation as well as improper handling of the beads. Furthermore, workflow issues can impede the use of counting beads in larger assays and 96-well plates. Here we introduce the next generation of absolute counting beads which overcome the impediments to reproducibility and repeatability in cell counting and enable easy workflows. We will compare this new format for cell counting to existing bead and instrument-based methods and demonstrate the improvements to the accuracy and reproducibility of cell counting. This improved accuracy will have important implications in research and development applications.

How to calculate absolute cell count

In instruments like the Invitrogen™ Attune™ NXT and Attune™ CytPix™ Flow Cytometers, absolute cell counts can be determined directly on the instrument itself. On most flow cytometers, absolute cell counting beads must be used as an internal calibration standard. Typically, at the end of a staining protocol a known quantity of beads is added to the sample. After running, the ratio of the counts of cells of interest to beads can be used to calculate the absolute cell count.

Figure 1. Example calculation of absolute cell count where the cell sample volume is 50 µL and the total number of beads added is 56,500.



$$Absolute\ Count\ \left(\frac{cells}{\mu L}\right) = \frac{Cell\ Count}{Bead\ Count} \times \frac{Total\ Beads}{Sample\ Volume\ (uL)}$$

$$Absolute\ Count\ \left(\frac{cells}{\mu L}\right) = \frac{3792\ cells}{10990\ beads} \times \frac{56{,}500\ beads}{50\ \mu L}$$

$$Absolute\ Count\ = 390\ \frac{cells}{\mu L}$$

Variability in absolute cell counting

Several factors have been identified that impact the reproducibility and repeatability of absolute cell counting. Many of these have been addressed in our new cell counting bead format.

Table 1. Factors impacting variability in determining absolute cell counts and how the new format of CountBright™ Plus addresses them.

Existing Absolute Cell Counting Products	New Format CountBright™ Plus Beads
• Pipetting accuracy (beads and cells) • Bead handling / aggregation (storage, mixing, pipetting) • Accuracy of supplied bead count (beads / 50 µL or tube) • Instrument maintenance and cleanliness • Experimental setup • Proper gating	• Improved bead QC → improved bead count accuracy • No aggregation during storage • No mixing, pipetting required • Improved experimental setup → Add and Read!

Bead properties impact absolute cell counting

Absolute cell counting beads need to be optimized to help ensure that their scatter and fluorescence properties allow for the most accurate determination of cell counts for populations of interest without interference. CountBright™ Plus beads have been optimized to have scatter properties in line with but outside of the scatter profiles of common cell types. Additionally, the fluorescence properties have been tuned to provide bright fluorescence in UV-NIR channels on both traditional and spectral flow cytometers. In our new format, we have improved the accuracy of our supplied bead counts to help ensure greater accuracy in determining cell counts.

Figure 2. Scatter profiles of Coulter® CytoTrol™ Cells and absolute counting beads on a Beckman Coulter CytoFLEX flow cytometer. (A) Cells with no beads. (B) Cells + Counting Bead B (~10% aggregation). (C) Cells + CountBright™ Plus Beads New Format (<3% aggregation). When comparing B and C, the aggregation in Counting Bead B is apparent. This aggregation will impact the repeatability and reproducibility of absolute cells counts as each aggregate is considered a single event. Supplied bead counts are typically based upon single beads only. Both beads are positioned well out of the scatter for the primary cell populations of interest, however SSC voltage had to be reduced for Counting Bead B.

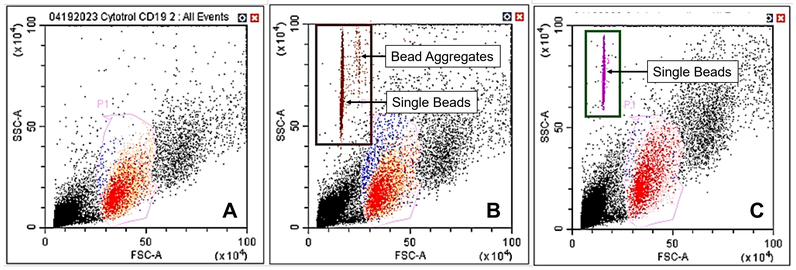


Figure 3. Fluorescence excitation (dotted lines) and emission spectra (solid) lines of the fluorescent dyes used in CountBright™ Plus beads. The dyes are efficiently excited by the most used laser lines (arrows) in flow cytometry and emit from 385 to 850-nm. These fluorescent properties make CountBright™ Plus beads ideal for both traditional and spectral flow cytometry.

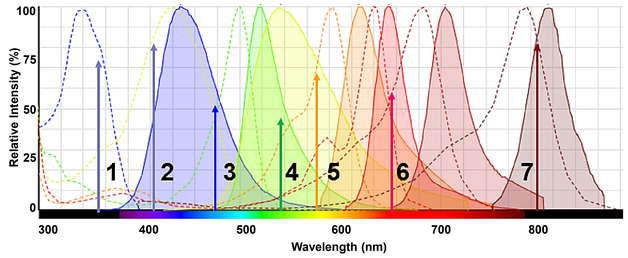
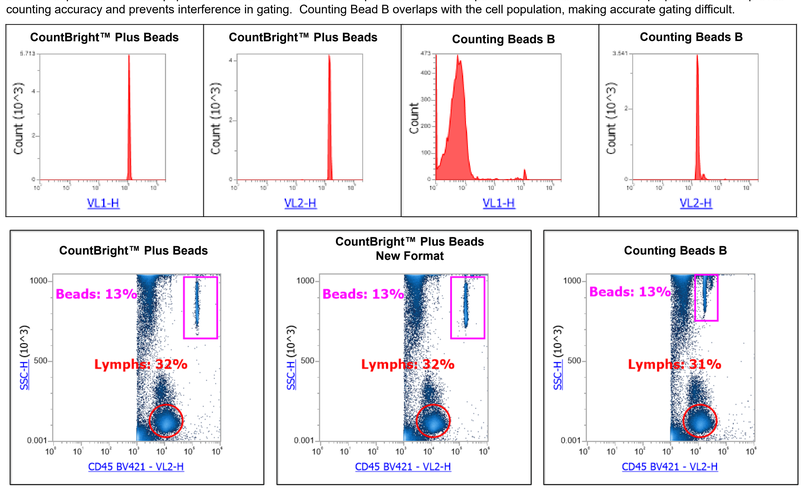


Figure 4. (Top) Histogram plots of both CountBright™ Plus beads and Counting Bead B in VL1 and VL2 channels on an Attune™ CytPix™ flow cytometer at the same PMT voltage. Counting Beads B show very little to no fluorescence in these channels. (bottom) CountBright™ Plus beads are well separated from cell populations in fluorescence vs. SSC plots due to their superior scatter and fluorescence properties. This improves counting accuracy and prevents interference in gating. Counting Bead B overlaps with the cell population, making accurate gating difficult.



Determination of absolute cell counts in lyse-no wash and no lyse-no wash workflows

To help assure that the new format CountBright™ Plus beads perform adequately in challenging scenarios, we took advantage of the superior abilities of the Attune™ CytPix™ for by Lyse-No Wash and No Lyse-No Wash workflows. The absolute cell counts of CD3 positive cells in whole blood were determined using three different counting beads. It was found that overall bead counts were similar across each of the bead types in these tests, though Counting Bead B was up to 11% lower in the No Lyse-No Wash protocol.

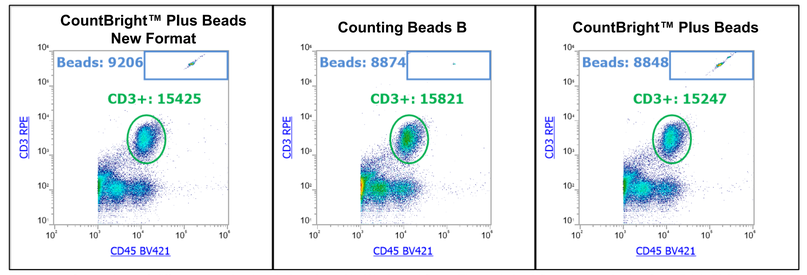


Figure 5. Comparison of different counting beads in a Lyse-No Wash workflow. The optimized fluorescence in the CountBright™ Plus beads allows for easier discrimination of beads from cells. All measurements were carried out on the same whole blood sample and using the bead counts from the supplier. Absolute CD3 positive cell counts: CountBright™ Plus New Format (82 cells / µL), CountBright™ Plus (86 cells / µL), Counting Beads B (86 cells / µL). Inset shows non-lysed red blood cells.

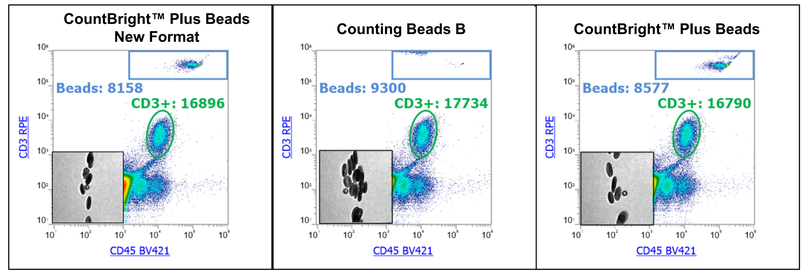


Figure 6. Comparison of different counting beads in a No Lyse-No Wash workflow. The optimized fluorescence in the CountBright™ Plus beads allows for easier discrimination of beads from cells. All measurements were carried out on the same whole blood sample and using the bead counts from the supplier. Absolute CD3 positive cell counts: CountBright™ Plus New Format (101 cells / µL), CountBright™ Plus (98 cells / µL), Counting Beads B (91 cells / µL). Inset shows non-lysed red blood cells.

Conclusion

CountBright™ Plus Absolute Counting Beads show exceptional scatter, single bead populations and fluorescence, allowing for improved accuracy in determining absolute cell counts. We have demonstrated this on a variety flow cytometers and workflows. To improve the accuracy of absolute cell counting, we have developed a new format for these beads that removes many of the factors that can lead to greater variability in cell counting. We have improved our methods for determining the number of beads per test, helping to improve overall accuracy. The new format prevents aggregation during storage and allows for an easy add-and-read workflow.

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