Cell counting

Improving cell counting accuracy and precision with automated, image-based counting

Abstract

Accurate, precise cell counts reduce experimental variability and enable consistent downstream assay inputs. Cell counting with a manual hemocytometer remains common but introduces operator-dependent variability through subjective focus and illumination settings and inconsistently applied inclusion/exclusion decisions. This application note consolidates best practices for sample preparation and instrument setup to allow optimal counting performance and highlights approaches to plan more controlled and sustainable cell counting experiments on the Invitrogen™ Countess™ 3 and Countess™ 3 FL Automated Cell Counters.

Introduction

Cell counting and viability measurement are a foundational step for cell culture maintenance and for quantitative workflows such as transfection, flow cytometry, high-content imaging, single-cell analysis, and functional screening. Errors in counts and viability measurements directly translate to incorrect plating density, altered growth kinetics, reduced comparability between samples and experiments, and increased cost due to failed experiments.

Accuracy: variability due to subjectivity from manual counts

Reliance on operator judgment contributes to errors in manual counting, especially in systems requiring manual focus and lighting. Operators must decide which objects to include or exclude as cells and, for viability, which cells are live versus dead. These subjective variables can drive broad user-to-user variation. Automated image-based cell counting reduces subjectivity by standardizing and automating focus, illumination, and analysis decisions using quantitative algorithms and consistent gating criteria (Figure 1).

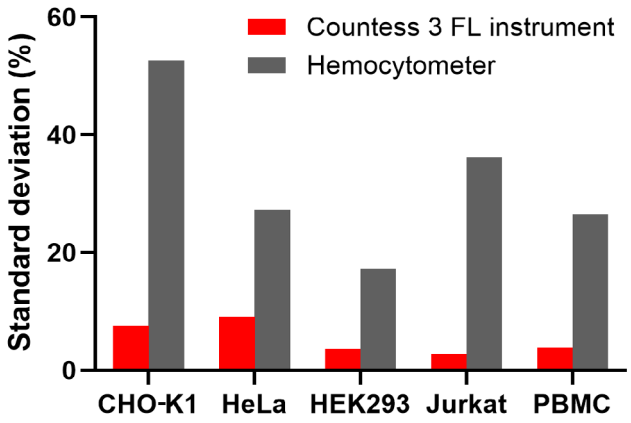

Figure 1. Comparison of user variability in cell counting with a hemocytometer and the Countess 3 FL instrument. Standard deviation of cell counts obtained by three different operators for multiple cell types shows that cell counting via a hemocytometer produces higher variability in counts compared to the Countess 3 FL instrument.

Sample preparation and instrument setup workflow

Accurate cell counting starts well before the sample is loaded into the counting chamber. The workflow below follows the practical sequence used in the Countess 3 and Countess 3 FL instruments and integrates key aspects from the accuracy and precision guidance including focus, staining, illumination, and gating.

Recommended workflow overview

1. Prepare a homogeneous single-cell suspension; mix immediately before aliquoting.

2. Stain consistently with colorimetric or fluorescence markers when required, following specified protocols; avoid particulate contamination, specifically with trypan blue (spin down, or pipette from top of vial).

3. Load the slide smoothly, avoiding bubbles; allow cells to settle (~20 seconds) to establish a uniform focal plane.

4. Select or create the appropriate instrument protocol; use autofocus and auto-lighting where available to minimize subjectivity.

5. Review cell identification and apply consistent gating criteria (size, circularity, intensity) across samples; save new instrument protocols for reuse.

6. Run technical replicates when precision matters; export results and images for traceability.

Counting area and the number of cells sampled

In practice, many researchers count too few cells during manual counting, which increases standard deviation and reduces confidence in the reported concentration. Counting a larger area improves consistency but adds time. The Countess 3 and Countess 3 FL instruments interrogate an area equivalent to nearly four traditional hemocytometer squares during the ~10-second acquisition (Figure 2), improving count-to-count consistency by reducing the impact of nonuniform cell distribution. To best count a representation of the starting sample, it's recommended to have at least 10 cells visible and counted in the field of view, resulting in a concentration of 1 x 10^5 cells/mL.

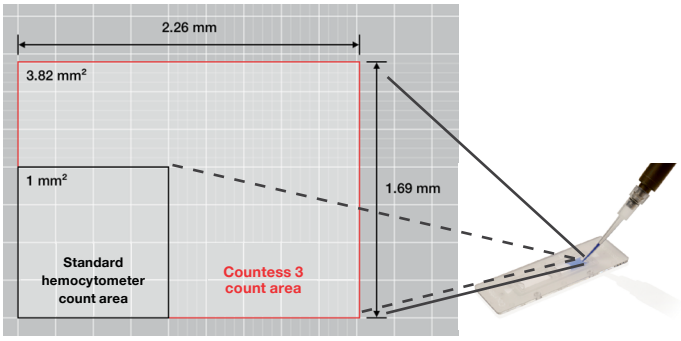

Figure 2. The counting area (in mm) of the Countess 3 instrument compared to that of a standard hemocytometer.

Replicates

Regardless of the counting platform or method, variance can be reduced by performing replicate counts. Before each count, thoroughly mix the sample, then withdraw an aliquot from the middle of the vial or tube to help ensure a representative, homogeneous sample and minimize variability due to pipetting. Automated cell counting makes replicates practical within routine lab timelines: multiple counts can be performed in the time required for a single careful manual count. Cells will die over time while not in their preferred culture medium and temperature. Measure replicates immediately, allowing for minimal differences in staining time (Figure 3).

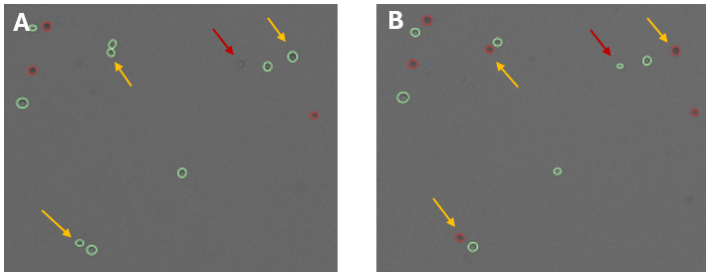

Figure 3. Cell death observed between replicate counts. **(A)** A peripheral blood mononuclear cell sample measured 3 minutes apart on the Countess 3 Automated Cell Counter shows that 3 cells identified as alive on the first count appear dead on the second count (yellow arrows). **(B)** The red arrow points to a cell that was not counted during the first count but counted as live on the second count.

Clumpy cells and aggregates

Obtaining accurate results with clumpy samples is challenging with manual counting and with some automated cell counting systems. A key requirement is the ability to identify cell boundaries within clumps to avoid undercounting. Cell identification algorithms on the Countess 3 and Countess 3 FL instruments can resolve singlets within complex clumps and report percent aggregation to help users detect and assess sample quality issues. Aggregates are clumps with 3 or more cells (Figure 4).

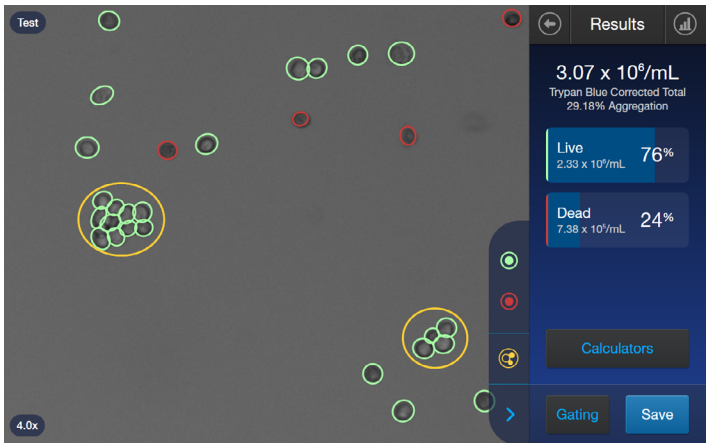

Figure 4. Accurate cell counts, even with clumpy samples. Example cell identification resolves cells in aggregates and reports percent aggregation under the total concentration.

Instrument focus

Focus is a primary driver of accurately counting and measuring viability. Out-of-focus images reduce edge contrast and can change apparent dye intensity, increasing misclassification risk. Standardizing focus (ideally via autofocus) improves repeatability across users and runs, particularly for trypan blue viability assays where subtle contrast differences separate live and dead cells (Figure 5).

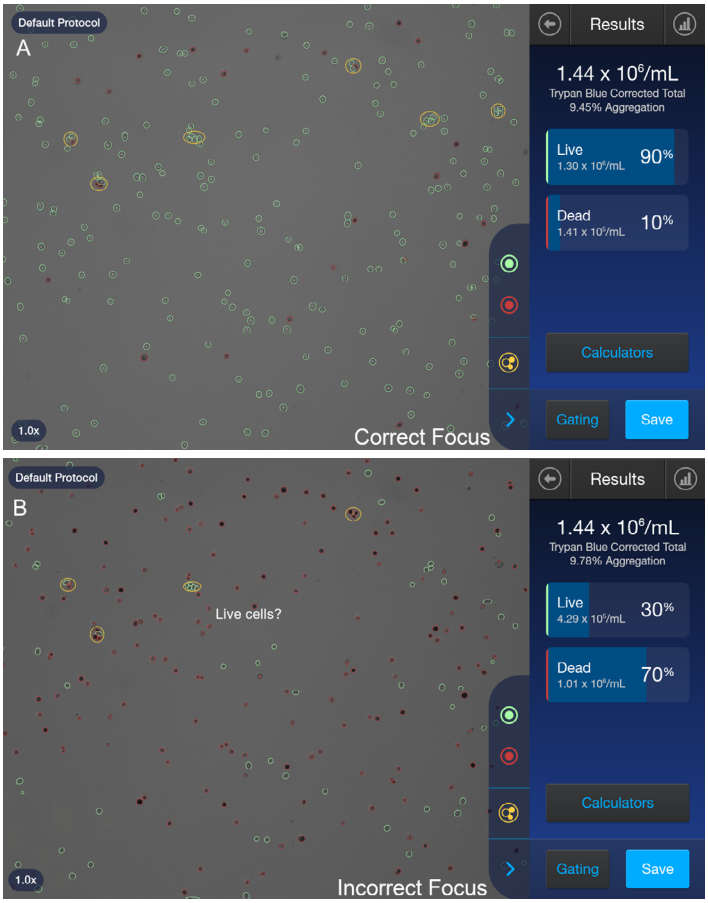

Figure 5. U20S cells counted on the Countess 3 Automated Cell Counter. (A) The live cells (circled in green) have bright centers and slightly darker borders when the focus is set properly. Dead cells (circled in red) will present a uniform dark staining pattern if trypan blue is present. **(B)** When an image is taken with incorrect focus, many live cells are identified as dead cells because the bright centers now appear dark.

Staining and light intensity

Staining consistency and illumination control are coupled: overly bright illumination can wash out contrast, while insufficient illumination can elevate background and obscure boundaries (Figure 6). For fluorescence-based counting, excitation intensity should maximize signal-to-noise ratio without saturating the image; consistent illumination supports stable thresholding across samples. Auto-lighting on the Countess 3 and Countess 3 FL instruments reduces user-driven variation by optimizing illumination for each acquisition (Figure 7). When performing experiments in which fluorescence intensity comparisons between samples are desired, the Countess 3 FL instrument enables fluorescence (FL) lock, disabling the auto-lighting, keeping the LED intensity consistent across samples.

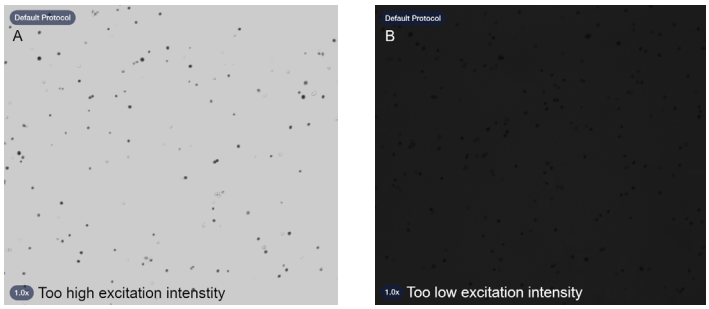

Figure 6. U20S cells counted on a Countess 3 Automated Cell Counter. (A) The brightfield light intensity is set too high. **(B)** The light intensity is too low.

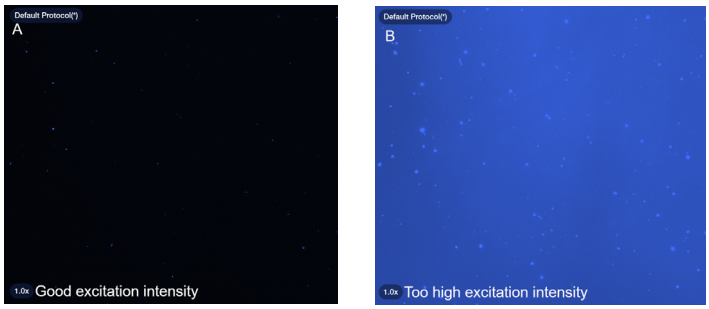

Figure 7. Jurkat cells stained with Invitrogen™ ReadyCount™ Nuclear Stain, counted with an Invitrogen™ EVOS™ DAPI Light Cube in a Countess 3 FL Automated Cell Counter. (A) The optimal fluorescent light intensity results in a good image. **(B)** Light intensity set too high results in elevated background.

Gating: apply consistent inclusion/exclusion criteria

Gating parameters (e.g., size, circularity, and intensity) are important controls for excluding debris and nontarget objects. To avoid introducing bias, apply the same gating strategy when comparing conditions, and save protocols so that criteria remain consistent across operators and experiments (Figure 8).

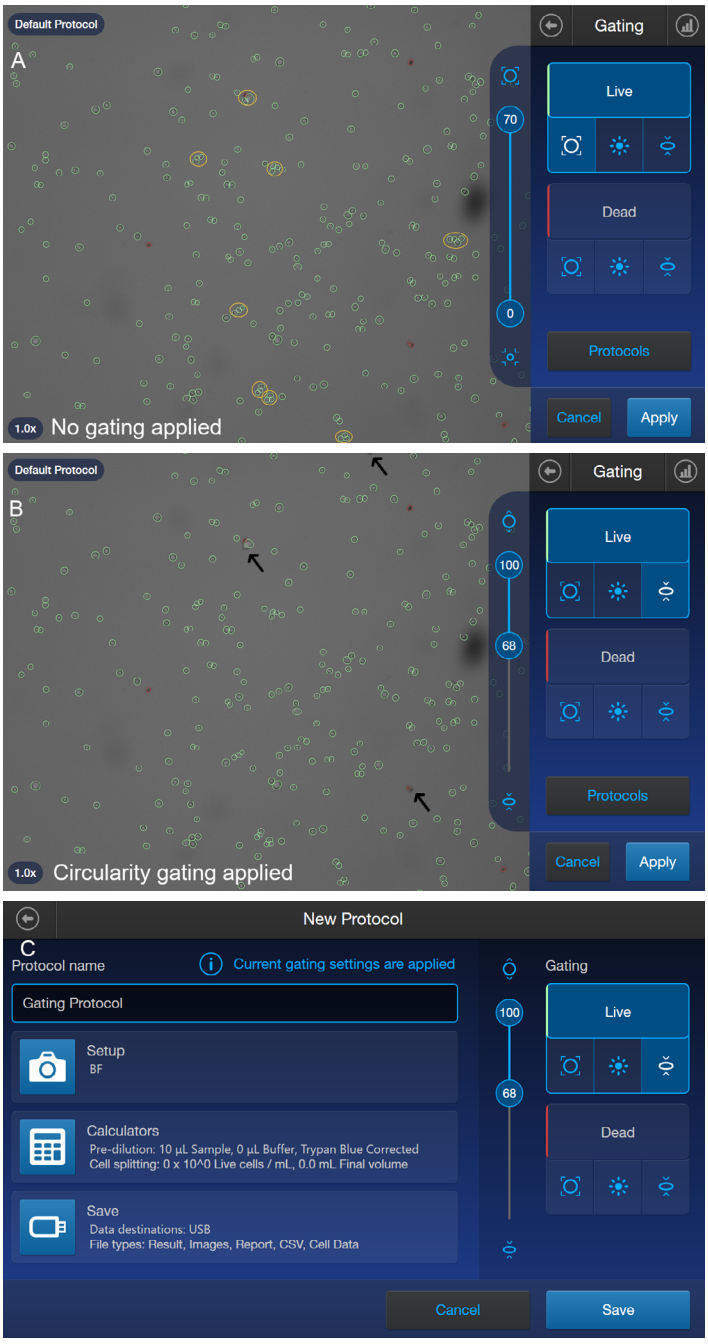

Figure 8. Gating adjustments can be made for each threshold parameter, size, brightness, and circularity. (A) No gating applied. **(B)** Circularity gating applied; note irregular cells not circled. **(C)** Gating settings are saved within a protocol.

Instrument-to-instrument variability

Understanding the primary contributors to variability in cell counting helps ensure accurate interpretation of results. The greatest variability is observed when replicates are taken from the same bulk suspension and counted independently. As discussed in the "Replicates" section, the biological replicate variability may result from inconsistent mixing, differences in pipetting technique, uneven cell distribution within the suspension, or cell death over time. Thorough mixing and minimizing time between counts help reduce this effect. In contrast, repeated measurements of the same sample on the same instrument show minimal variability (coefficient of variation (CV) of ~1%), demonstrating strong repeatability. Minor differences between technical replicates may reflect slight cell movement, small autofocus or auto-lighting adjustments, or limited cell death during the measurement interval (Figure 9).

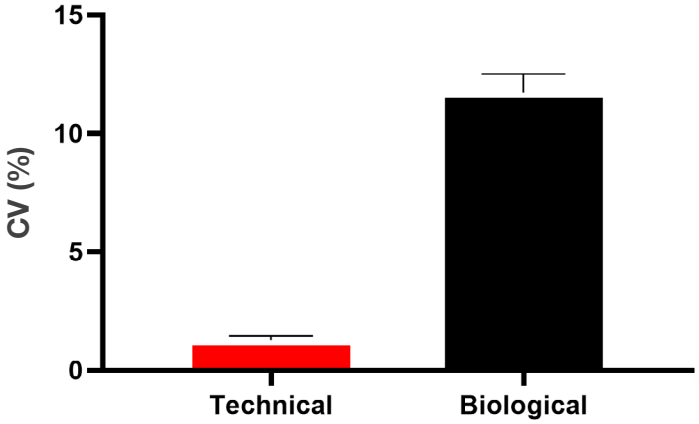

Figure 9. Accuracy and precision in the same instrument. Seven cell types were stained and counted using the same protocols and reagents, in triplicate, on the same instrument with both technical and biological replicates. The low CV for technical replicates (<2%) indicates high measurement precision when the same slide is counted repeatedly. In contrast, CVs of ~11% for biological replicates of the same sample reflect variability introduced during sample handling and preparation, including pipetting inconsistency, incomplete mixing, and changes in cell viability between measurements.

When the same prepared sample is counted on two different instruments, variability is lower than what is seen between aliquots but higher than technical replicates. These differences are primarily due to each instrument imaging a slightly different field of view within the chamber of the slide. If cells are not evenly distributed on the slide, small differences in the number of cells captured may occur, particularly at lower cell concentrations where fewer total cells are counted and statistical variation increases proportionally (Figure 10). Collectively, these findings demonstrate that sample preparation and handling contribute more to variability than instrument performance, and that Countess 3 and Countess 3 FL Automated Cell Counters support consistent and reproducible results across instruments.

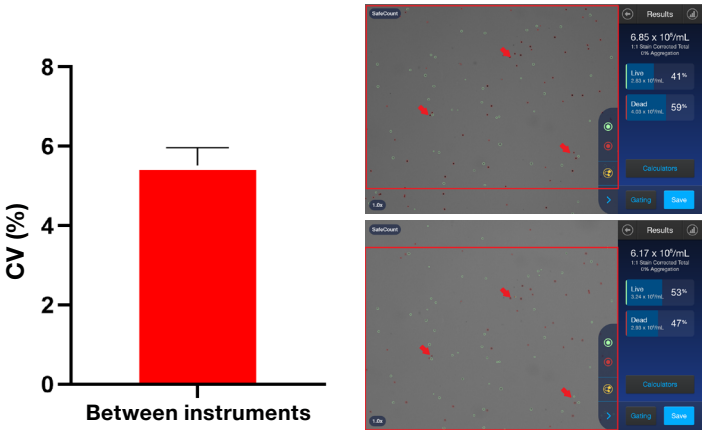

Figure 10. Accuracy and precision between instruments. Seven cell types were stained and counted using the same protocols and colorimetric reagents and analyzed on three different instruments. The CV of ~5% across instruments indicates a high degree of precision between instruments. Minor variability arises because each instrument captures slightly different fields of view on the same slide as shown in the images. Overlapping regions (red box) and identical cells (red arrows) are shown for reference, while nonoverlapping areas contribute to small differences in total cell counts and resulting concentration.

Summary

Accurate and precise cell counting is essential for experimental reproducibility and for controlling downstream variability in quantitative workflows. The highest-impact aspects to reliable cell counting remain disciplined sample preparation, standardized image acquisition, and consistent inclusion/exclusion criteria through defined gating strategies.

By combining automated autofocus, auto-lighting, and quantitative image analysis with robust consistency, the Countess 3 and 3 FL Automated Cell Counters reduce both operator- and system-driven variability. Together, these attributes support reliable cell counting at scale, enabling comparison of results across users, instruments, and experiments with confidence.

Additional application notes

Peripheral blood mononuclear cell (PBMC) counting

Counting of single cells and nuclei for scRNA-Seq and scATAC-Seq

Automated cell counting compared to manual cell counting

Product	Quantity	Cat. No.
Countess 3 FL Automated Cell Counter	1 instrument	AMQAF2000
Countess 3 Automated Cell Counter	1 instrument	AMQAX2000
SafeCount Cell Viability Stain	2 x 1 mL vials	A40008024
Trypan Blue Stain	2 x 1 mL vials	T10282
ReadyCount Red Dead Cell Stain	1 mL vial	A49903
ReadyCount Blue Nuclear Stain	1 mL vial	A49904
ReadyCount Green/Red Viability Stain	1 mL vial	A49905
Countess Cell Counting Chamber Slides and Holder, disposable	50 slides, 2 x 1 mL vials of trypan blue stain	C10228
	500 slides, 20 x 1 mL vials of trypan blue stain	C10312
	50 slides, trypan blue stain not included	C10283

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