The importance of accurate cell counting in flow cytometry and cell sorting

Introduction

Many flow cytometry and cell sorting protocols recommend a specific cell concentration or range of concentrations for optimal staining and analysis. Incorporating cell counting into the flow cytometry workflow is a simple way to help increase experimental success and potentially save valuable time and money. This procedure enables:

- Confirmation of having a sufficient number of cells for the protocol
- Determination of the optimal amount of staining reagent
- Knowledge of the amount of cells lost during staining
- Verification of staining efficiency

Are you sure you have the optimal concentration of viable cells prior to staining?

For many flow cytometry staining protocols, the starting cell number or concentration is critical to achieving optimal staining and may dictate the appropriate amount of staining reagents, incubation time, and reaction volume. With an accurate cell count before starting a staining protocol, you can use the appropriate recommended amounts of cells and stains, avoiding waste of valuable reagents and time (Figures 1 and 2).

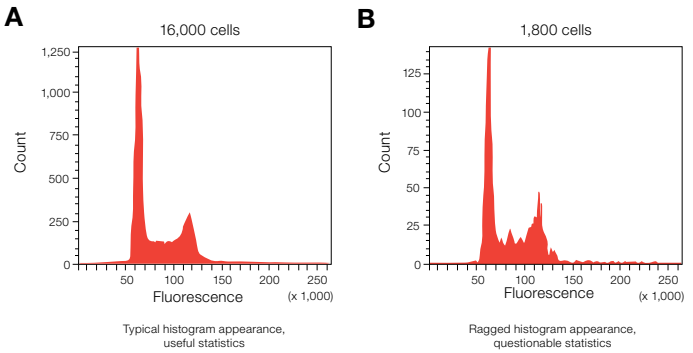

Figure 1. Having a sufficient number of cells is required for optimal cell cycle analysis with flow cytometry. Live Jurkat cells were labeled with Invitrogen™ Vybrant™ DyeCycle™ Orange Stain, which is a cell-permeant dye that fluoresces upon binding to DNA. Different numbers of cells were then acquired for cell cycle analysis. **(A)** Acquisition of 16,000 cells produced a cell cycle histogram with a typical appearance and useful statistics, while **(B)** acquisition of only 1,800 cells produced a cell cycle histogram with a ragged appearance and questionable statistics.

What is the appropriate amount of stain needed for the sample?

Applying too little stain can lead to problems such as weak signals, while overstaining can increase background and cause bleed-through of signal into other fluorescence channels, both of which can affect proper discrimination of fluorescent cell populations and waste valuable reagents. Automated cell counters that measure viability can confirm that cells are healthy and alive before you proceed further in a staining protocol.

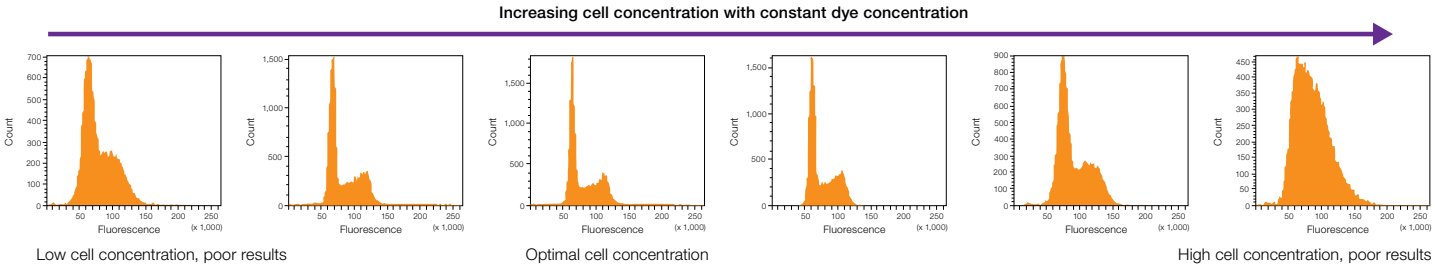

Figure 2. Effect of cell concentration on cell cycle analysis by flow cytometry. Different concentrations of live Jurkat cells were labeled with a constant concentration (10 µM) of Vybrant DyeCycle Orange Stain. Using the same concentration of stain produced poor cell cycle histograms with both low and high cell concentrations, while staining with the optimal cell concentration of 1 x 10^6 cells/mL gave the proper cell cycle histogram.

Did your staining achieve the expected results?

Having a way to quickly count cells and examine fluorescence after staining is also valuable. Cell loss is inevitable in many staining protocols, so a cell count after staining can determine how many cells were lost and if a concentration step is required before analysis. With over 20 interchangeable light cubes to choose from, the Invitrogen™ Countess™ II FL Automated Cell Counter also enables you to quickly visualize the fluorescence of cells to determine the efficiency or quality of cell staining or transduction for a wide variety of dyes and fluorescent proteins, not only GFP and RFP. With other methods such as manual counting, you can determine the total number of cells but not how well the cells are transduced or fluorescently stained; the fluorescence feature of the Countess II FL Automated Cell Counter enables accurate assessment of transduction or staining efficiency (Figure 3), which can differ even if the total cell number does not change.

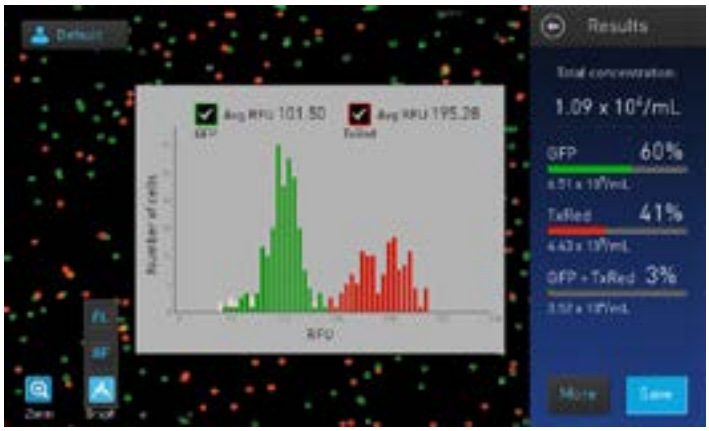

Figure 3. Gating based on fluorescence intensity. Two fluorescence colors are present, each of which can be gated by size, brightness, circularity, and fluorescence intensity. The dim cells (around 50 RFU) have been excluded from the GFP-positive count, as indicated by the light-colored portion of the bars.

The Countess II FL Automated Cell Counter allows you to quickly examine a small sample of cells before analyzing your full cell sample in flow cytometry for a large cell population. Although the Countess II FL Automated Cell Counter is not intended to replace your flow cytometer, as it does not provide data from a large number of events, these analysis methods are complementary to each other. The Countess II FL Automated Cell Counter can be used to quickly screen a representative sample to determine the quality of fluorescence and data trends before more comprehensive flow cytometry analysis (Figure 4). A quick check of cellular fluorescence prior to analysis can confirm that the cells are properly stained or expressing fluorescence as expected before they are analyzed further.

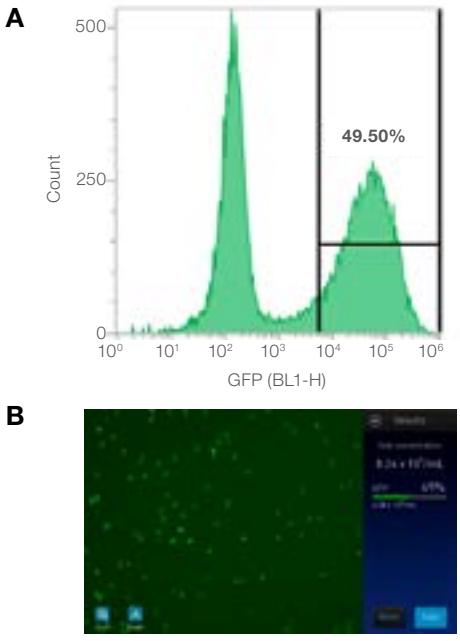

Figure 4. Fluorescent cell counts similar to those obtained by flow cytometry can be easily acquired with the Countess II FL Automated Cell Counter, with a single light cube. U2OS cells were transduced using Invitrogen™ CellLight™ Nucleus-GFP, BacMam 2.0, and allowed to incubate for 36 hours. The cells were evaluated for GFP on **(A)** a flow cytometer and **(B)** the Countess II FL Automated Cell Counter. The proportions of transduced cells determined by the two methods are nearly identical.

Manual vs. automated counting

Compared to traditional methods such as manual counting using a hemocytometer and microscope, the Countess II FL Automated Cell Counter provides significant benefits in counting accuracy and time savings. These benefits help to increase experimental confidence and allow more time for flow cytometry acquisition and analysis.

Reduced user variability for increased counting accuracy

When manually counting cells using a hemocytometer and a microscope, count-to-count variability of a single sample by an experienced technician is typically 10% or more. Counting variability between multiple technicians commonly exceeds 20%. Reliance on operator judgment, regardless of the cell counting method, contributes to this variability. Light intensity and focus settings on a microscope and some automated cell counters are subjective and therefore can contribute to variability. Individual users must also determine which objects to include and exclude in the cell count, as well as decide which cells will be counted as alive or dead. The number of subjective variables in manual counting can thus lead to wide variation in results between users, resulting in inconsistency in the number of cells stained and analyzed in flow cytometry. Automated cell counters minimize the subjective nature of manual counting as well as user-to-user differences in total cell counts (Figure 5).

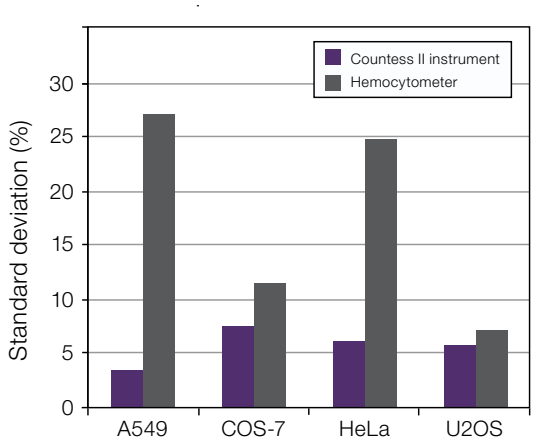

Figure 5. User variability when counting with a hemocytometer compared to using a Countess II instrument. Identical samples of A549, COS-7, HeLa, and U2OS cells were counted by three different operators using a Countess II instrument and then manually with a hemocytometer and microscope. The user-to-user variability is much higher for the hemocytometer than for the Countess II instrument.

Time savings

The additional time it takes to manually count cells (up to 5 minutes) compared to counting with the Countess II FL Automated Cell Counter (10 seconds) is often overlooked as an added cost. An individual counting 5 slides per day (2 samples per slide) can save about 10 hours per month by switching to an automated counter with a reusable slide. The time savings increase to about 15 hours per month when using disposable slides (Figure 6). This additional time may allow cells to be counted at multiple points throughout a flow cytometry workflow without increasing the time it takes to complete an experiment.

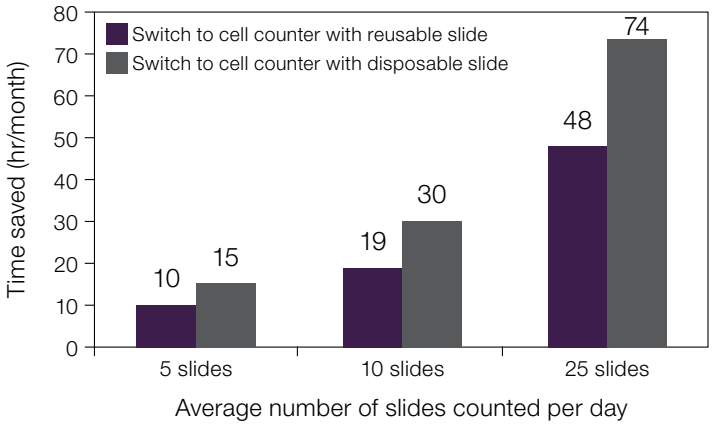

Figure 6. Estimated hours per month saved by switching from manual cell counting to an automated cell counter.

Options for automated cell counting

Counting cells and measuring viability is quick and easy with the Countess II or II FL Automated Cell Counter—simply stain a 10 µL sample of cells with trypan blue, pipet the stained cells into a chamber slide, then allow the instrument to autofocus and count to obtain the total cell concentration along with the concentration and percentage of live and dead cells (Figure 7). Alternatively, many flow cytometry users today utilize single-channel viability markers, like Invitrogen™ LIVE/DEAD™ Fixable Dead Cell Stains, which can also be observed with the Countess II FL Automated Cell Counter (Figure 8). These stains can help users avoid potential fluorescence quenching artifacts due to trypan blue staining.

Figure 7. Counting of peripheral blood mononuclear cells (PBMCs) using the Countess II Automated Cell Counter. Counting and viability measurements are done using trypan blue staining.

A

B

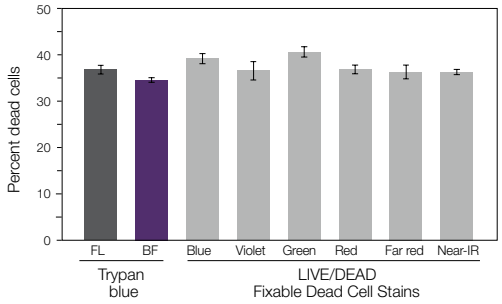

Figure 8. Cell viability detection using single-color viability assays. Live and ethanol-killed Jurkat cells were mixed (~2:1 live:dead), stained with trypan blue or with various colors of LIVE/DEAD Fixable Dead Cell Stains, and analyzed on the Countess II FL Automated Cell Counter equipped with the appropriate Invitrogen™ EVOS™ Light Cube. **(A)** A cell population treated with LIVE/DEAD Fixable Green Dead Cell Stain (Cat. No. L34969) for 30 min at 37°C and analyzed on the Countess II FL instrument equipped with the GFP EVOS Light Cube. **(B)** A dead cell count by trypan blue staining was determined by fluorescence (FL) or brightfield (BF) imaging. For the LIVE/DEAD stains, the dead cell count was determined using the fluorescence channel. The percentages of dead cells differ from the theoretical 2:1 starting ratio (33%) because the "live" population contained some dead cells.

Conclusion

The ability to easily perform multiple cell counts to determine cell concentration or verify staining efficiency—all before scheduling time on a costly core facility instrument or performing a large-scale experiment—can save time and money. Since most other methods for counting cells are time-consuming or require expensive equipment and calibration, cell counting is often not performed throughout a protocol. The Countess II and II FL Automated Cell Counters offer a quick and simple way to determine cell number and examine cell health and viability. Since total cell counts, viability, apoptosis, and fluorescence can be measured in as little as 10 seconds, these key parameters can easily be confirmed at several points throughout a flow cytometry workflow. The Countess II and II FL Automated Cell Counters can give you the confidence to proceed through your workflow and help ensure a successful experiment.

Protocols for cell counting

Examining cell number, viability, and fluorescence is quick and easy with the Countess II FL Automated Cell Counter.

Materials

- Countess II FL Automated Cell Counter (Cat. No. AMQAF1000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess II FL Reusable Slide (Cat. No. A25750)
- Invitrogen™ Trypan Blue Stain (Cat. No. T10282) or fluorescent stain

Protocol for obtaining total cell count and viability

1. Mix 10 µL of cell sample with 10 µL of trypan blue and pipet 10 µL of the stained sample into a Countess chamber slide.
2. Insert the slide into the sample port of the Countess II FL instrument to initiate autofocus.
3. Press the "Count" button.
4. Optimize results if needed by using the gating options for size, brightness, and circularity. Viewing the histogram while gating can help you determine the appropriate settings for your sample.
5. Record the concentrations and percentages of total, live, and dead cells.
6. Optional: Use the built-in dilution calculator to determine the appropriate cell dilution for your flow cytometry staining protocol.

Protocol for analyzing fluorescence

1. Stain the cell sample (Table 1) according to the flow cytometry staining protocol, or obtain cells expressing fluorescence.
2. Pipet 10 µL of the fluorescent cell sample into a Countess chamber slide.
3. Insert the slide into the sample port of the Countess II FL instrument to initiate autofocus.
4. Adjust light intensities to minimize fluorescence background.
5. Press the "Count" button.
6. Optimize results if needed by using the gating options for size, brightness, circularity, and fluorescence intensity. Viewing the histogram while gating can help you determine the appropriate settings for your sample.
7. Record the concentration and percentage of fluorescent cells.
8. Optional: Use the built-in dilution calculator to determine the appropriate cell dilution for your flow cytometry staining protocol.

Table 1. Dyes and light cubes commonly used with the Countess II FL cell counter for viability and apoptosis determination.

Dye	Light cube	Cat. No.
Viability dyes		
ReadyProbes Cell Viability Imaging Kit, Blue/Green	DAPI and GFP	R37609
ReadyProbes Cell Viability Imaging Kit, Blue/Red	DAPI, and RFP or Texas Red	R37610
LIVE/DEAD Viability/Cytotoxicity Kit	GFP, and RFP or Texas Red	L3224
Propidium Iodide ReadyProbes Reagent	RFP	R37108
SYTOX Green Nucleic Acid Stain	GFP	S7020
SYTOX Red Dead Cell Stain	Cy®5	S34859
7-Aminoactinomycin D (7-AAD)	Texas Red or Cy5	A1310
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	DAPI	L34965
LIVE/DEAD Fixable Green Dead Cell Stain Kit	GFP	L34969
LIVE/DEAD Fixable Red Dead Cell Stain Kit	Texas Red	L23102
Apoptosis dyes		
CellEvent Caspase-3/7 Green Detection Reagent	GFP	C10423
SYTOX Red Dead Cell Stain	Cy5	S34859

invitrogen

Application notes

Find application notes with protocols for viability, apoptosis, transfection, and PBMC counting using the Countess II instruments.

thermofisher.com/countess

Convince your lab

We can help address key areas to consider when purchasing an automated cell counter. Download the prewritten letter, petition, and application note demonstrating the benefits of an automated cell counter.

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White paper

Improve your cell counting results with this white paper discussing the four pillars of accurate cell counting.

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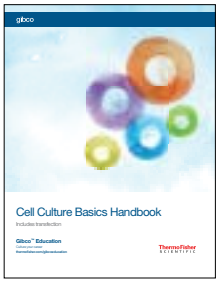

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Ordering information

Product	Quantity	Cat. No.
Countess II FL Automated Cell Counter	1	AMQAF1000
Countess II Automated Cell Counter	1	AMQAX1000
Countess Cell Counting Chamber Slides	50	C10228
Countess II FL Reusable Slide	1	A25750
Countess II FL Reusable Slide Holder	1	AMEP4746

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