

Counting of single cells and nuclei on Countess Automated Cell Counters for scRNA-Seq and scATAC-Seq



Cell analysis

Introduction

Single-cell RNA sequencing (scRNA-Seq) and single-cell sequencing assay for transposase-accessible chromatin (scATAC-Seq) technologies enable new insights into gene expression and regulation by providing data resolution at the single-cell level. To obtain high-quality single-cell data, protocols for scRNA-Seq and scATAC-Seq require an accurate count of viable suspended single cells or nuclei as input, with minimal presence of cellular aggregates and dead cells. The Invitrogen™ Countess™ 3 FL Automated Cell Counter is a dependable solution for accurately counting cells and nuclei, evaluating their viability, and assessing aggregation. To ensure optimal performance of the Countess 3 FL cell counter in such protocols, this application note provides a best practices guide.

Materials

- Countess 3 FL Automated Cell Counter (Cat. No. AMQAF2000)
- Countess Cell Counting Chamber Slides (Cat. No. C10228) or Countess Reusable Slide (Cat. No. A25750)
- Fluorescent markers for viability such as the Invitrogen™ ReadyCount™ Green/Red Viability Stain (Cat. No. A49905) (see the [Fluorescent cell viability assays on Countess Automated Cell Counters](#) application note for more reagents)
- Invitrogen™ Trypan Blue Stain (Cat. No. T10282) (optional)
- Your preferred scRNA-Seq or scATAC-Seq reagent kits (such as from 10x Genomics)

Instrument setup

1. Turn on the Countess 3 FL Automated Cell Counter and install the appropriate Invitrogen™ EVOS™ light cubes for the stains being used. Most commonly, the ReadyCount Green/Red Viability Stain requires GFP and Texas Red™ 2.0 light cubes.
2. Install the appropriate slide holder for either the disposable or reusable slide.
3. Obtain a Countess disposable or reusable slide.

Single-cell preparation and counting

Follow sample preparation protocols suggested for the chosen scRNA-Seq or scATAC-Seq kits, starting with tissue samples, cells in suspension, or tissue culture cells.

Minimizing cell aggregates

To maximize the output of single-cell data in scRNA-Seq experiments, it is crucial to ensure that the starting samples do not contain cell aggregates. This is important to ensure that individual cells can be partitioned and barcoded during the single-cell library preparation process. If input samples contain cell aggregates, all cells in a given aggregate will be partitioned together and labeled with the same barcode, and extra filtering will be required during data analysis to remove these aggregate-derived barcodes. To optimize maximum single-cell data output, you can confirm that your starting sample has <5% aggregates using the Countess 3 Automated Cell Counter.

Protocol for assessing cell aggregates in brightfield (BF) mode

1. Load 10 μL of cell suspension into a Countess chamber slide.
2. Insert the Countess chamber slide into a Countess 3 instrument.
3. Confirm that “BF” is checked and press “Count” (fluorescence (FL)-based count is not checked).
4. In the top-right corner, observe that the percentage aggregate number is less than 5% (this number reflects how many total cells are within aggregates compared to the total number of cells).
 - If your sample has more than 5% aggregates, include repeated pipetting, vortexing, and/or filtering steps in your sample preparation protocol to break down aggregates before continuing.

Optional: To find aggregates of only live cells, perform steps 1–4 using a 1:1 dilution of sample with Trypan Blue Stain in BF mode.

5. On the results screen, gate out the dead cells by pressing “Gating”.
6. Highlight “Dead”.
7. Adjust the gates until there are no red circles on the screen.
8. Press “Apply”.

Accurate counts of viable cells using FL-based count mode

When performing scRNA-Seq, methods require accurate viable cell counts. If starting samples are counted inaccurately, systems may clog or reagent concentrations may not be optimal for the needed chemistry to occur. To optimize accurate viable cell counting, use fluorescent stains such as the ReadyCount Green/Red Viability Stain (Cat. No. A49905) and count using the FL-based count mode of the Countess 3 instrument with software update 3. The FL-based count mode detects all fluorescent objects and leads to more accurate counts for samples when the BF image has cellular debris or cells out of focus. See the [Fluorescent cell viability assays on Countess Automated Cell Counters](#) application note for more reagents. When using fluorescent stains, first create a protocol to ensure that the result obtained considers dye dilution factors.

Creating an FL-based count protocol

1. Press “Protocols”.
2. Press “Create”.
3. Name your protocol.
4. Press “Setup”.

5. Check the light cubes that will be used (GFP and Texas Red light cubes), and if you’d like to capture a brightfield image, check the BF box.
6. Check “FL-based count”.
7. Press “Apply”.
8. Press “Calculators”.
9. Press “Pre-dilution calculator”.
10. Input the volumes of the cell suspension and fluorescent dye (i.e., 10 μL for “Sample volume” and 10 μL for “Buffer volume” for ReadyCount stains).
11. Press “Apply”.
12. Optional: Adjust the saving location and gating parameters if desired by selecting “Save” and utilizing gating features on the right of the screen.
13. Press “Save”.
14. Press “Load” to use.

FL-based count protocol

1. Mix cells with fluorescent dye: if using ReadyCount stains, mix 10 μL cells with 10 μL stain.
2. Incubate cells for the specified time: if using ReadyCount stains, incubate for at least 2 min.
3. During incubation, select or create a protocol on the Countess 3 FL instrument.
4. Load 10 μL of the cell and stain suspension onto a Countess chamber slide.
5. Wait at least 20 sec for the cells to settle on the slide.
6. Insert the Countess chamber slide into the Countess 3 FL instrument.
7. Confirm that the desired light cubes and “FL-based count” are checked.
8. Press “Count”.

The total concentration reported is based on the combination of all fluorescent cells. Each light cube reports the total number of cells detected for that light cube. Cells that are fluorescent with both light cubes are reported as dual-positive cells (if dual-positive cells are present, the percentage of cells will not add up to 100% because dual-positive numbers are also included in the total count for single-positive cells for the same light cube). If using ReadyCount Green/Red Viability Stain, green cells reflect live cells while red cells reflect dead cells.

Gating

When preparing cell suspensions for scRNA-Seq, particularly from tissue sections, it is common to encounter red blood cells or components such as cellular debris, which can lead to inaccurate cell counts. Using the FL-based counting mode provides an accurate count because it only counts fluorescent objects. Thus, some objects seen in the BF image may not be counted if they are not fluorescent. If any objects observed have low-level fluorescence that you do not want included in your count, adjust the gating parameters to the desired fluorescence threshold.

Increasing speed with fluorescence (FL) lock

Cell preparation for scRNA-Seq must occur as fast as possible to ensure that cells are healthy and viable. The Countess 3 FL instrument will autofocus on the sample and use fluorescent cube automatic lighting to provide an accurate count. If you need to increase the cell counting speed, utilize the FL lock function to establish a set fluorescent light value rather than requiring the instrument to automatically light each sample. Do this on the first sample by pressing “Adjust” and selecting the FL lock icon.

Optimal concentrations for counting

The Countess 3 FL instrument is able to count samples in the concentration range of 1×10^4 – 1×10^7 cells/mL, but it is most accurate at 1×10^5 – 2×10^6 cells/mL. When using the Countess 3 FL instrument to determine concentrations less than 1×10^5 cells/mL, take the average of three independent counts to improve accuracy. If these counts are variable, concentrate your cells by gentle centrifugation and resuspension in a lower volume. If your sample is more than 2×10^6 cells/mL, dilute the sample prior to counting.

Preparation and counting of single nuclei

scATAC-Seq is a technique that involves the characterization of isolated nuclei to investigate the accessibility of chromatin regions in the genome. The Countess 3 FL Automated Cell Counter supports counting of nuclei and enables counting objects as small as 3 μ m. Follow nuclear isolation protocols for desired downstream applications with the addition of these suggested best practices with the Countess 3 FL instrument.

Reagents

Fluorescent stains are highly recommended for assessing and counting suspensions of nuclei, because of their increased accuracy compared to trypan blue, which is prone to overcounting nuclei due to the staining of cellular debris. ReadyCount fluorescent stains are benchtop stable and optimized for easily staining and counting nuclei and nucleated cells on the Countess 3 FL instrument.

- ReadyCount Green/Red Viability Stain (Cat. No. A49905)—includes a mixture of acridine orange and propidium iodide
- ReadyCount Red Dead Cell Stain (Cat. No. A49903)—includes propidium iodide
- ReadyCount Blue Nuclear Stain (Cat. No. A49904)—includes Hoechst™ stain
- LIVE/DEAD Viability/Cytotoxicity Kit (Cat. No. L3224)—includes ethidium homodimer-1

See the [Fluorescent cell viability assays on Countess Automated Cell Counters](#) application note for more reagents.

Protocol

Assay setup and focus adjustment:

1. Follow the nuclear isolation or sequencing kit protocols.
2. Create a protocol on the Countess 3 FL instrument for the desired fluorescent stain (see “Creating an FL-based count protocol” on page 2).
3. Insert a Countess slide containing the fluorescently stained nuclei.
4. On the setup screen, inspect the fluorescent nuclei (if using propidium iodide, this would be the Texas Red light cube).
 - If needed, adjust focus and FL lighting to better identify the nuclei.
 - Please note: the focus is based on the BF image.
5. Press “Adjust”.
6. Press “Focus”—this will load the BF image.
7. Drag the focus bar up or down to change focus.
8. Press “FL light cube” to confirm that focus adjustment improved the FL image.

FL lighting:

9. Manually adjust the lighting to better identify the nuclei.
10. Optional: To ensure the same lighting is used for all subsequent counts of nuclei, press the FL lock icon.
11. Press “Apply”.
12. Press “Count”.

Gating:

13. On the results screen, focus your attention on the FL light cube layer for nuclei and the corresponding circle indicating the number of cells counted.
14. Press “Gating”.

15. Using buttons for size, brightness, and circularity, adjust desired parameters to specifically identify nuclei and not background FL objects.
16. Optional: To save these new settings, press “Protocols”.
 - a. Press “Create”.
 - b. Confirm that current gating settings are applied to the protocol.
 - c. Create a protocol name.
 - d. Press “Save”.
 - e. To return to the results screen, press “Cancel”.
17. Press “Apply” to apply the gating settings to the current count.
18. Press “Save” to save the results and count.
19. To ensure the accuracy of the counts, we recommend that you perform replicate counts (2 or 3 counts that can be reproduced) using the same protocol and gating parameters.

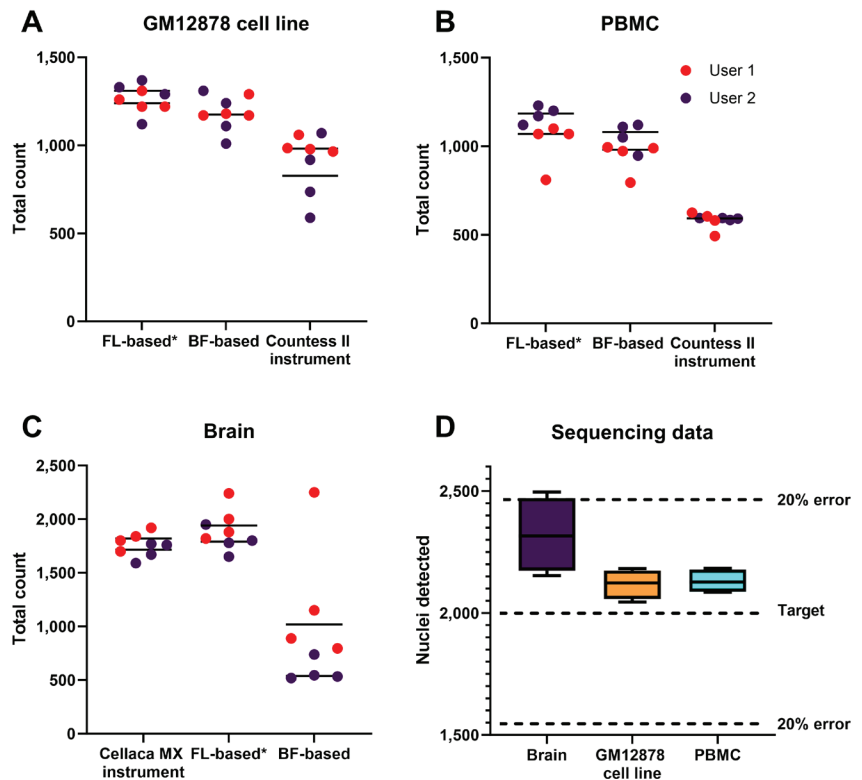


Figure 1. Counting of nuclei on the Countess 3 FL Automated Cell Counter using FL-based counting resulted in accurate counts, verified via sequencing. FL-based counts of nuclei isolated from the GM12878 cell line (A) and PBMCs (B) counted more nuclei than BF-based or Countess II instrument counts. (C) Isolation of nuclei from brain tissue produced accurate FL-based counts, comparable to counts from the Cellaca™ MX High-Throughput Automated Cell Counter. (D) Next-generation sequencing (NGS) confirmed accurate loading of 2,000 target nuclei, obtained from FL-based counts. *FL-based counts available on Countess 3 FL instrument using software update 3. Data obtained by 10x Genomics R&D.

Results

Figure 1 shows examples of results obtained from counting nuclei isolated from two different cell types and brain tissue.

Conclusion

The Countess 3 FL Automated Cell Counter is a dependable solution for accurately counting cells and nuclei to perform single-cell analysis. Using this guide to accurately count suspensions of viable single cells or nuclei, with minimal presence of cellular aggregates and dead cells, will help produce high-quality single-cell data.

Ordering information

Product	Quantity	Cat. No.
Countess 3 Automated Cell Counter	1 each	AMQAX2000
Countess 3 FL Automated Cell Counter	1 each	AMQAF2000
Countess 3 Automated Cell Counter Starter Package	1 each	A49865
Countess 3 FL Automated Cell Counter Starter Package	1 each	A49866
ReadyCount Green/Red Viability Stain	100 assays	A49905

Product	Quantity	Cat. No.
ReadyCount Red Dead Cell Stain	100 assays	A49903
Countess Reusable Slide	1 unit	A25750
Countess Cell Counting Chamber Slides	50 slides	C10228
LIVE/DEAD Viability/Cytotoxicity Kit	1 kit	L3224
ReadyCount Blue Nuclear Stain	100 assays	A49904
Trypan Blue Stain	2 x 1 mL	T10282

Find out more at thermofisher.com/countess

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