



BLOG

How to Count Cells

Nuclei Counting Tips for Single-Cell Sequencing : A Practical Guide to Getting It Right

From dye selection to cell size data — the last QC checkpoint before sequencing



biosystems

by ALIGNED GENETICS

Introduction

Counting a nuclei suspension before scRNA-seq or snRNA-seq library prep is always a bit nerve-wracking. Most sequencing library prep protocols call for input within a defined concentration range, so getting the concentration or purity wrong here can send the sequencing cost and time downstream to waste. Where does nuclei counting actually sit in the overall workflow? Our application note lays out the single-cell sequencing workflow in five stages:

Tissue Collection → Isolated Cell (Nuclei) Preparation → Sequencing Library Preparation → Sequencing → Analysis.

Counting falls at the very end of the Isolated Cell (Nuclei) Preparation stage — right before Sequencing Library Preparation. It's the last checkpoint in sample prep, and the step that determines the input concentration going into library prep.

Why Isolate Nuclei Instead of Whole Cells for Sequencing?

So why isolate nuclei instead of whole cells for counting in the first place? Single-cell sequencing is, in principle, designed for intact whole cells. But many samples — tissues like brain, adipose, and skeletal muscle — are physically difficult to fully dissociate down to the single-cell level. Pushing dissociation too hard can stress the cells enough to alter their gene expression relative to the original tissue state, a phenomenon known as a dissociation artifact. Frozen or archived tissue and biobank samples present another challenge: cell membranes are often already compromised by the freeze-thaw process, making it difficult to recover intact cells at all. In these cases, isolating nuclei instead of whole cells and running snRNA-seq (single-nucleus RNA-seq) becomes the practical alternative — it's gentler on the tissue while still capturing reliable cell-type information, which is why it has become widely adopted.

Why Nuclei Quality Needs to Be Checked, Not Just Counted

Here's the catch: 'nuclei isolated' and 'nuclei cleanly isolated' are not the same thing. During isolation, some nuclei retain residual cytoplasm or membrane fragments, and nuclei often clump into clusters. Counting these as-is can inflate the apparent concentration, and downstream, it can introduce ambient RNA contamination during library prep — undermining the reliability of the final sequencing data. That's why, at the counting step, it matters not just how many nuclei you have, but how cleanly they were separated.

How Nuclei Quality Is Typically Assessed

That judgment call is harder than it sounds. Nuclei, like dead cells, stain positive with membrane-impermeable dyes, so fluorescence alone often can't distinguish partially isolated nuclei, fully isolated nuclei, and dead cells from one another. In practice, labs typically combine three approaches: first, membrane-impermeable dyes (PI, EthD-1, etc.) to confirm the cell membrane has actually been removed; second, cell size data — since nuclei are smaller than intact cells — to gauge how complete the separation is; and third, direct inspection of brightfield images to check for debris or clustering. Together, these three approaches catch what fluorescence alone tends to miss.

This article draws on our own application note data to walk through practical checkpoints — organized around these three axes (dye, size, and imaging) — for improving nuclei counting accuracy in single-cell workflows. All experimental data and parameter settings shown were generated in-house on the [LUNA-FX7™](#) Automated Cell Counter; if you're using a different instrument, we recommend focusing less on the exact settings and more on what to check for.

Tip 1. Start With the Right Dye Combination

Assessing nuclei quality starts with choosing the right dye combination. In theory, an intact cell should respond only to membrane-permeable dyes like AO or Calcein AM, while a nucleus with no membrane left should respond only to membrane-impermeable dyes like PI or EthD-1.

In practice, when we compared four combinations — AO/PI, AO/EthD-1, Calcein AM/PI, and Calcein AM/EthD-1 — in U937 and 3T3 cells, AO paired with either PI or EthD-1 most accurately distinguished the viability of intact cells from isolated nuclei. Calcein AM, on the other hand, depends on esterase activity, so its signal intensity varied considerably by cell type: 3T3 cells showed almost no Calcein AM signal, while only about two-thirds of U937 cells did — meaning some intact cells risked being missed entirely.

Dye Combinations Supported by the LUNA-FX7™

The [LUNA series](#) supports only the Green (AO, etc.) and Red (PI, EthD-1, etc.) fluorescence channels — it does not support Blue-wavelength dyes such as DAPI or Hoechst 33342.

For nuclei counting, use an AO/PI or AO/EthD-1 combination. Intact cells stain green (AO); isolated nuclei stain red (PI or EthD-1).

Tip 2. Use Cell Size Data to Judge How Complete the Separation Is

Because nuclei have lost their membrane and cytoplasm, they measure significantly smaller than intact cells. This size difference lets you quantify how far along the isolation process actually is. Below is the average cell size data we measured on the [LUNA-FX7™](#) after AO/PI staining, from two of our own experiments (see application note).

Cell line	Intact cells	Partially isolated nuclei	Fully isolated nuclei
U937	12.5 µm	-	5.6 µm
3T3	17.8 µm	-	7.2 µm
HL60	13.4 µm	10.2 µm	8.3 µm

The U937 and 3T3 values come from an experiment comparing cell size before and after lysis, while the HL60 values come from a separate experiment that measured undissociated, partially isolated, and fully isolated samples at each stage. Notably, HL60 nuclei that were only partially isolated averaged 10.2 µm — right between the fully isolated nuclei (8.3 µm) and the intact cells (13.4 µm). In other words, checking average cell size alongside the counting result lets you catch particles that were counted as nuclei but were actually still only partially separated. Because the [LUNA-FX7™](#) extracts size information from brightfield images rather than fluorescence intensity, it's less prone to staining-related size distortion — making this a more reliable check.

Tip 3. Telling Dead Cells and Isolated Nuclei Apart

Dead cells and isolated nuclei both have a damaged or absent membrane, so both stain equally red with membrane-impermeable dyes like PI. In other words, fluorescence signal alone can't tell the two apart. This is where brightfield-based size data comes in. In HeLa cells, for example, the average size of intact cells versus isolated nuclei was clearly distinguishable — 13.4 µm vs. 6.4 µm — and in HEK293 cells, 13.3 µm vs. 11.7 µm. The [LUNA-FX7™](#) reports this size data alongside the fluorescence result, letting you confirm — both visually and numerically — whether a PI-positive signal is coming from a dead cell or an isolated nucleus.

Tip 4. Check for Clumping and Clusters Before Sequencing

When dissociating tissue into a single-cell or nuclei suspension, it's common for some cells to remain stuck together in clusters. In one real example, a sample of incompletely dissociated AsPC-1 cells had roughly 9% of the population still in clusters, with viability at only 56% — and was judged unsuitable for downstream processing. The cluster histogram feature lets you check this cluster percentage numerically before moving on to library prep.

For heavily clumped samples, re-dissociation is generally the first thing to try. But if a tissue simply won't dissociate cleanly into single cells no matter what, switching to nuclei isolation and nuclei counting is worth considering as an alternative. Because counting is based on the nuclear membrane rather than the cell boundary, it may reduce the risk of undercounting caused by clumping. That said, this depends on cell type and sample condition, so we'd recommend testing both approaches side-by-side on your own samples before committing to one.

Tip 5. Working With PFA-Fixed Nuclei

When nuclei are fixed with PFA for long-term storage, the outcome can depend on which dye combination you use. The AO/PI combination gives a consistently strong PI signal regardless of fixation status or staining time, so it works reliably on both fresh and fixed nuclei.

AO/EthD-1, on the other hand, tends to show weaker red fluorescence in fixed nuclei, and extending the staining time doesn't necessarily bring the signal back up. In this case, raising the [LUNA-FX7™](#)'s exposure setting from the default (5) to 8 got us signal levels comparable to AO/PI. (If you're using a different instrument, try a similar adjustment to its exposure or gain settings.)

Tip 6 (Bonus). FFPE-Derived Nuclei Need a Different Staining Strategy

Nuclei isolated from formalin-fixed, paraffin-embedded (FFPE) tissue need to be handled differently from fresh tissue. Deparaffinization and the extra isolation steps involved generate a lot of debris, and membrane-permeable dyes like AO bind non-specifically to this debris, creating noise.

What to Watch for When Staining FFPE-Derived Nuclei (LUNA-FX7™)

AO/PI is not recommended for FFPE-derived nuclei — AO's non-specific binding to debris compromises accuracy.

Instead, use a single membrane-impermeable dye such as PI or YOYO-1, and optimize the [LUNA-FX7™](#)'s LED settings.

Raising the RF LED for PI from the default 5 to 7 improves the signal, while lowering the GF LED for YOYO-1 from the default 5 to 2 reduces background noise. (On other instruments, try a similar adjustment to the corresponding fluorescence channel intensity settings.)

Tip 7. Simplify the Workflow With Settling Time and SpectraSlide® AP-1

Small, low-density cells or nuclei — splenocytes, for example — can lose counting accuracy if imaged immediately after loading onto the slide, before enough particles have settled into the focal plane. Time-lapse imaging showed that waiting about 1–2 minutes after loading, before counting, noticeably improves image sharpness and contrast — making this brief settling time an important checkpoint when working with small particles.

If you want to cut down on dye prep altogether, the AO/PI-precoated [SpectraSlide® AP-1](#) is another option worth considering. When tested against the standard [PhotonSlide™](#) on splenocytes and isolated nuclei, it showed strong correlation — $R^2 = 0.9997$ for viability, and $R^2 = 0.998$ (splenocytes) and $R^2 = 0.9995$ (nuclei) for concentration — delivering comparable accuracy without any separate dye prep. Live (green) and dead (red) signals stayed reliably distinguishable even in debris-heavy or low-viability samples.

Accurate nuclei counting isn't the end goal in itself — it's the last QC gate standing between you and an expensive sequencing investment. Choosing the right dye combination, checking separation completeness with cell size data, tracking cluster percentage, and using settings appropriate to fixation status — working through these four checkpoints in order is the most practical way to cut down on failed sequencing runs.

Frequently Asked Questions (FAQ)

Q. Should I use AO/PI or AO/EthD-1?

A. For freshly isolated nuclei, either combination works fine. If you'll be working with PFA-fixed nuclei, though, AO/PI is the more stable choice. If you need to use AO/EthD-1 on fixed nuclei, we recommend raising the [LUNA-FX7™](#)'s exposure setting to 8.

Q2. Q. My viability reading is close to 0% — did the experiment fail?

A. That's actually the expected signal when nuclei isolation has gone well. Since the membrane has been removed, membrane-impermeable dye penetrates freely, pushing viability toward 0%. Don't judge by that number alone — check whether the average cell size falls within the expected nuclei size range as well.

Q3. Can the LUNA-III™ also do nuclei counting?

A. The [LUNA-III™](#) is a brightfield-only automated cell counter with no fluorescence channel, so it doesn't support AO/PI-based nuclei counting. AO/PI or AO/EthD-1-based nuclei counting requires the [LUNA-FX7™](#).

Reference

1. Application Note — Fluorescent Dyes and Automated Cell Counters: Towards Reliable Nuclei Quality in Single Cell Genomics. Logos Biosystems.
2. Application Note — Automated Cell Evaluation for Single-Cell RNA-seq Analysis. Logos Biosystems.
3. Application Note — Assessing Single Membrane-Impermeable Dyes for Nuclei Assessment Derived from Kidney FFPE Tissue Using the LUNA-FX7™. Logos Biosystems.
4. Application Note — Evaluation of the SpectraSlide® AP-1 for Accurate Counting of Splenocytes and Isolated Nuclei. Logos Biosystems.
5. Krishnaswami SR et al. Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. Nat Protoc. 2016;11:499–524.