BLOG

How to Count Cells

Why Is Counting Primary Cells So Difficult?

PBMC, stem cells, immune cells — cell-type-specific challenges and practical solutions

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Introduction

You finish isolating PBMCs, run a cell count, and find yourself wondering whether to trust the number. The count is lower than expected, viability seems oddly high, or two back-to-back measurements come out significantly different. For anyone working with primary cells, this kind of uncertainty is familiar.

Unlike established cell lines, primary cells carry variables that are difficult to control: donor-to-donor biological variation, mixed cell populations, and stress introduced during the isolation process. A counting protocol that works perfectly with a cell line will often fall apart with primary cells.

This post covers the main counting challenges for key primary cell types — PBMCs, stem cells, and immune cells — and what you can practically do about each one.

Why Primary Cell Counting Is Harder

Most automated cell counters are optimized around cell lines like HeLa or CHO — uniform in size, consistent in morphology, and well-behaved in suspension. Primary cells break every one of those assumptions.

Problem 1 — Wide size range

PBMCs alone span two distinct populations: lymphocytes (7–10 μm) and monocytes (15–20 μm). Set the size threshold to capture lymphocytes and the counter will classify monocytes as debris; set it for monocytes and small debris gets counted as cells. There is no single threshold that cleanly captures both.

Problem 2 — Clumping and cluster formation

iPSCs grow as colonies and are difficult to bring into true single-cell suspension. Activated immune cells form clusters. When aggregates are present, the counter either drastically undercounts — or reads a clump of cells as a single large cell. Either way, the result is unreliable.

Problem 3 — Isolation stress

The isolation process itself — enzymatic digestion, density gradient centrifugation, mechanical dissociation — puts cells under stress. Membrane integrity can be transiently compromised, causing cells that are actually viable to take up dead-cell stains. This is one of the most common reasons viability readings come out lower than expected.

Problem 4 — Trypan blue limitations

Trypan blue is quick and convenient, but particularly unreliable with primary cells. It classifies cells with transient membrane damage as dead, and its brightness-based discrimination is thrown off by large size variation. In mixed populations like PBMCs, undercounting is common.

Cell-Type-Specific Challenges and Approaches

Not all primary cells are difficult in the same way. The dominant problem varies by cell type, and so does the solution.

Cell Type	Main Challenge	Recommended Approach	Counting Difficulty
PBMC	Mixed monocyte/lymphocyte sizes; residual RBCs	Density gradient + AO/PI fluorescence staining	★★★★☆
Stem cells (iPSC / MSC)	Colony formation, aggregates, small cell size	Single-cell dissociation + image-based verification	★★★☆☆
NK cells	Size heterogeneity; morphology shifts upon activation	Fluorescence staining + adjusted size threshold	★★★★☆
Dendritic cells (DC)	Dendritic projections, cluster formation	Dissociation to single cells required before counting	★★☆☆☆
Neurons	Neurites obscure cell boundaries; fragile	Nuclei counting method recommended	★★☆☆☆

PBMC Counting — Common, But Trickier Than It Looks

PBMCs are among the most frequently counted primary cells — used in immunology research, CAR-T manufacturing, vaccine studies, and more. They are also the source of some of the most persistent counting errors.

Residual red blood cells

Even after Ficoll density gradient centrifugation, small numbers of red blood cells often remain. RBCs are small (6–8 μm) and anucleate, making it easy for automated counters to confuse them with debris — or vice versa. Running an RBC lysis step before counting, or using fluorescence staining to confirm the presence of nuclei, helps reduce this interference.

Why AO/PI fluorescence staining works better

Acridine Orange (AO) stains nucleic acids in live cells with green fluorescence; Propidium Iodide (PI) enters membrane-compromised cells and stains their DNA red. Because viability is determined by fluorescence signal rather than cell size, this combination is far more accurate for size-heterogeneous populations like PBMCs. One key point: in AO/PI results, the green signal (AO+) represents total cell count, and the red signal (PI+) represents dead cells. Viability is calculated as AO+ / (AO+ + PI+).

Count as soon as possible after isolation

PBMC condition changes over time after isolation. Counting within 30 minutes of completing the isolation is strongly recommended. Leaving cells on ice for extended periods or allowing them to sit at room temperature accelerates membrane degradation, resulting in artificially low viability readings.

Stem Cell Counting — Aggregation Is the Main Enemy

iPSCs and MSCs present different counting challenges, but both come down to biology that resists single-cell suspension.

iPSC — Colonies must be dissociated before counting

Because iPSCs grow as colonies, dissociation into single cells is a required step before counting. EDTA or Accutase are commonly used, but if dissociation conditions are off — either too gentle or too aggressive — aggregates remain or cells are over-damaged. Count immediately after dissociation, and pass the sample through a 40 μm cell strainer to remove remaining clumps before measurement.
Reviewing the count image is equally important. Image-based automated cell counters capture a snapshot alongside the count result, letting you immediately see whether significant aggregation remains — something a number alone cannot tell you.

MSC — Large and inconsistent in size

MSCs are substantially larger than most cell lines (15–30+ μm) and become more variable in size with increasing passage number. Default size thresholds on most cell counters (typically set for 7–15 μm) will miss a significant fraction of MSCs. Adjusting the upper size threshold to match the actual cell population, or switching to fluorescence staining to reduce sensitivity to size variation, are the practical fixes here.

Immune Cell Counting — Account for Activation State

NK cells, T cells, and dendritic cells (DCs) all change in size and morphology depending on their activation state. Counting parameters optimized for resting cells will not apply accurately to activated cells.

NK cells — Size increases upon activation

Activated NK cells are larger than resting NK cells and have increased granule content. Within the same culture, count results can differ meaningfully depending on when during the activation process the measurement is taken. Count at the time point relevant to your experiment, and use image-based review to monitor the size distribution at each stage.

Dendritic cells — Irregular morphology causes undercounting

Mature DCs develop long dendritic projections that make their shape highly irregular. Many automated cell counters use circularity as part of the cell recognition algorithm, and DCs with well-developed projections can be excluded as non-cells. Where possible, review the count image and adjust thresholds accordingly, or consider nuclei-based counting approaches.

Neuron Counting — When Whole-Cell Counting Isn't the Answer

Neurons are among the most difficult cell types for conventional automated counting. Axons and dendrites extending from the soma make cell boundaries undefined, and mechanical dissociation severe enough to create a single-cell suspension causes substantial cell damage.

Wear a mask — non-negotiable

Oral Mycoplasma is the single most common route of contamination. Always wear a mask during cell culture work and minimize unnecessary talking in front of the biosafety cabinet. It sounds simple, and it is — but it is also the most effective single preventive measure.

Recommended approach for neuron counting

For neurons, nuclei counting is more practical than whole-cell counting. Staining nuclei with DAPI or Hoechst 33342 and counting them by image-based analysis avoids the cell boundary problem entirely. This approach is also widely used in single-cell RNA sequencing (scRNA-seq) sample preparation workflows.

Choosing a Staining Method — Primary Cell Comparison

Staining method choice directly affects counting accuracy for primary cells. Trypan blue is convenient, but for cells with high size variation or significant isolation stress, fluorescence-based staining is consistently more reliable.

Staining Method	Detection Mode	Accuracy	Notes for Primary Cells
Trypan Blue	Brightfield	★★☆☆☆	Fast and simple, but error-prone with primary cells. Sensitive to size variation.
AO/PI	Fluorescence (Green/Red)	★★★★★	Direct fluorescence discrimination of live vs. dead cells. Standard for primary cells.
AO only	Fluorescence (Green)	★★★★☆	Provides cell size and concentration data. Cannot distinguish dead cells.
Propidium Iodide (PI)	Fluorescence (Red)	★★★☆☆	Selectively stains membrane-compromised (dead) cells. No live-cell information when used alone.

For most primary cell counting applications, AO/PI is the most balanced choice. In heterogeneous populations like PBMCs and immune cells, it delivers consistently more accurate results than trypan blue.

Pre-Count Checklist for Primary Cells

This is one of the most common misconceptions in cell culture. Penicillin has no activity against Mycoplasma, as it targets cell wall synthesis and Mycoplasma have no cell wall. Streptomycin likewise has only limited effectiveness for Mycoplasma prevention or elimination. A different class of antibiotic is required.

Check these before you count

- **Know your cell's size range** — confirm the counter's detection range (μm) covers the cells you are working with
- **Choose the right staining method** — if size variation is high or isolation stress is expected, use AO/PI fluorescence staining
- **Check for aggregation** — review the image before counting. If significant clumping is present, filter through a 40 μm strainer first
- **Count promptly after isolation** — especially for PBMCs: count within 30 minutes of completing isolation
- **Record your dilution factor** — document the exact dilution used and apply it correctly in the final calculation
- **Always review the count image** — a number without an image leaves no way to catch systematic counting errors

Closing Thoughts

Primary cell counting is harder than cell line counting because biological variability and isolation-process variation feed directly into the result. No single standardized protocol will cover every cell type, and finding the right staining method, size threshold, and timing for each cell population takes deliberate effort.

The most practical starting point comes down to three things: know the size range of the cells you are working with, consider switching to AO/PI fluorescence staining, and make it a habit to review count images alongside numerical results. With these in place, the most common sources of primary cell counting error are largely addressable.

Frequently Asked Questions (FAQ)

Q1. My trypan blue and AO/PI counts for PBMCs are very different. Which should I trust?

A. AO/PI fluorescence staining is generally more accurate. Trypan blue classifies cells based on membrane integrity, and cells with transient membrane damage from the isolation process are often misclassified as dead. AO/PI binds directly to nucleic acids, which reduces this type of misclassification. For size-heterogeneous populations like PBMCs, AO/PI consistently delivers more reliable results.

Q2. My iPSC counts vary significantly between runs. What's going wrong?

A. The most common cause is inconsistent dissociation. If single-cell separation is incomplete, residual aggregates lead to systematic undercounting. Standardize your dissociation time and reagent concentration, add a 40 μm strainer step before counting, and run at least three replicates under identical conditions to assess your CV (coefficient of variation). Getting CV under control is the first step toward a stable iPSC counting protocol.

Q3. Viability readings for my immune cells are consistently too low. Where should I look?

A. Check your timing, storage temperature, and how long cells sit before staining. Storing cells on ice for extended periods or at refrigerator temperature can increase membrane permeability, allowing PI to enter cells that are actually viable. As a general rule, count as soon as possible after isolation and keep samples at room temperature during the process.

Reference

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