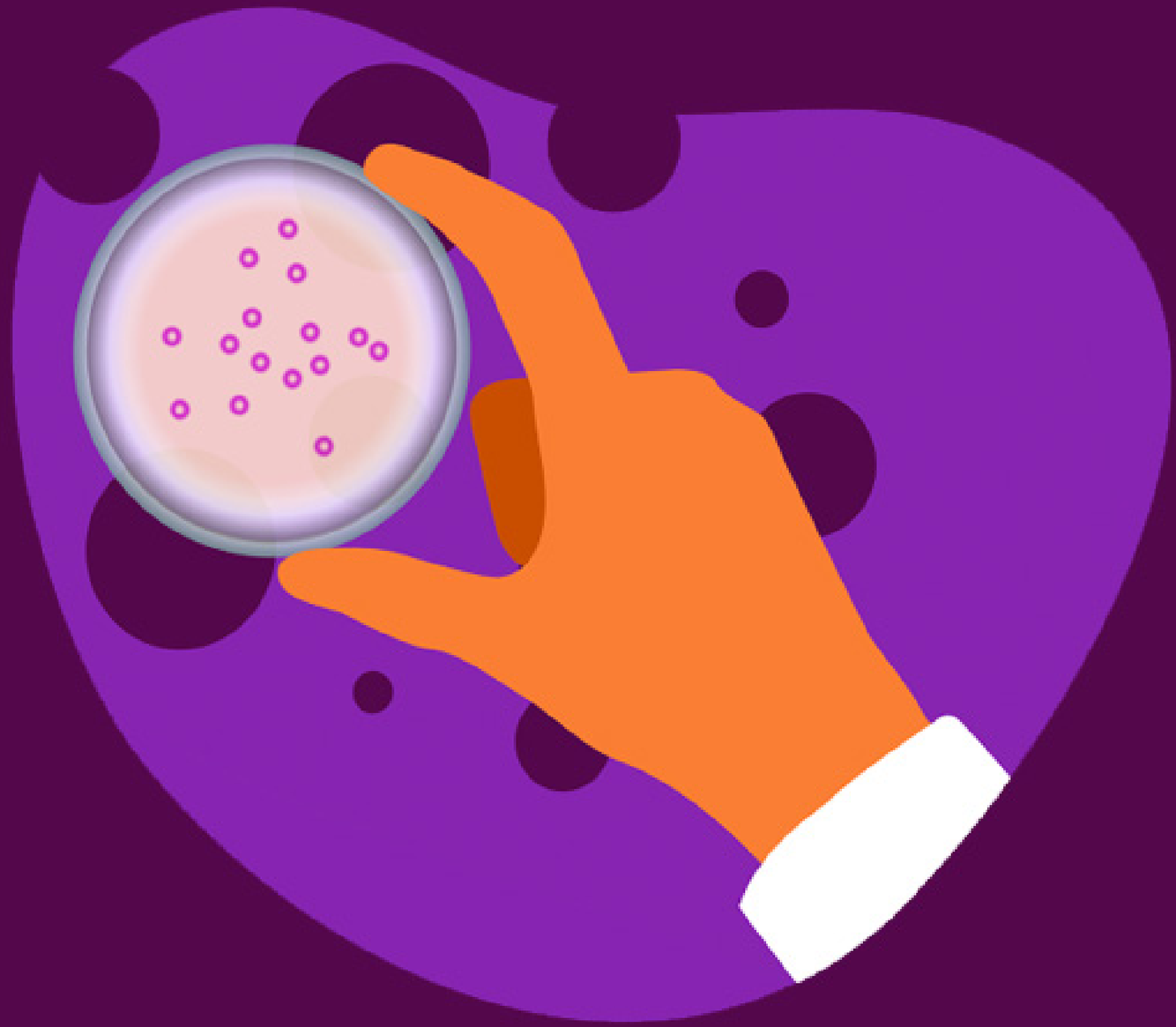
10 key factors to improve your cell counting results



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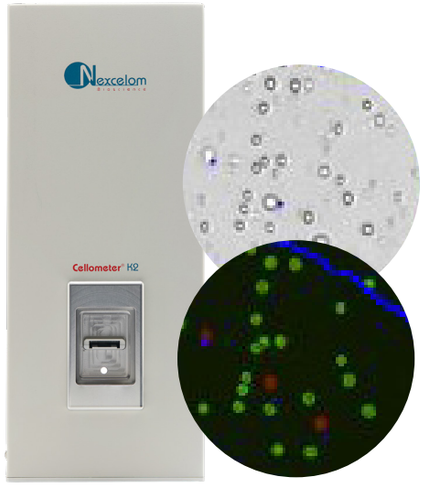
Introduction



As cell and gene therapies have progressed in the last decade, the quality requirements for cell samples have increased because they are involved directly with patient treatment. This necessitates the replacement of traditional cell counting methods with modern, high-quality alternatives.

Cell counting is crucial for products reviewed by multiple FDA centers, as well as all phases of cell and gene therapy and regenerative medicine drug evaluation. Modernization of cell counting measurements is critical to support the development and manufacturing of these advanced biological products.

This Ebook provides best practices for selecting, comparing, and evaluating cell counting methods as detailed in several articles created in collaboration by the US Food and Drug Administration (FDA) in collaboration with the US National Institute of Standards and Technology (NIST) and the International Organization for Standardization (ISO).

1 Challenges for high-quality cell counting are rooted in the nature of live cells.

There are many challenges that can hinder high-quality cell counting. Live cells are complex, dynamic and heterogeneous, and are sustained by many varieties of nutrient media. There is also a wide range of biological sample types, such as immortal cell lines, tumor digests, yeast, and bacteria, which can be involved in various bioprocessing steps. Finally, there is no live cell reference material for traditional evaluations of measurement accuracy defined by the Chemical Manufacturing Control (CMC) and the Validation of Analytical Procedures [ICH Q2 (R1)].[4] These challenges can cause variations in cell counting results and due to the lack of reference standards, often the accuracy of the tested cell counting methods cannot be determined (Figure 1).

Accuracy and precision matter

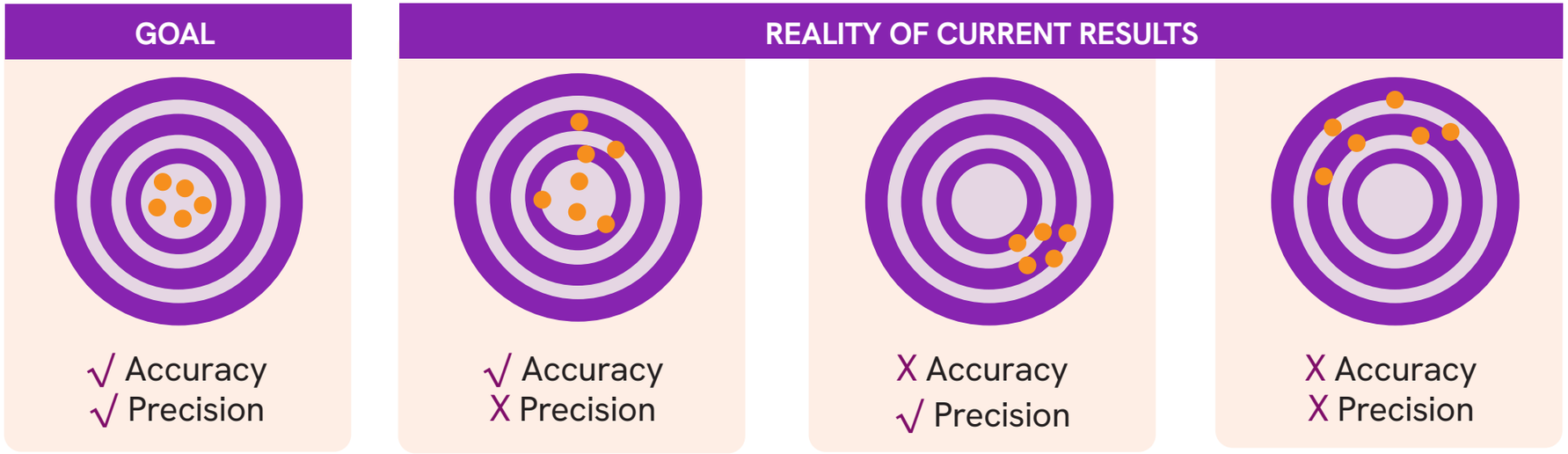


Figure 1: Cell counting targets that demonstrate differences between accuracy and precision. Due to the lack of reference standards, we may only be able to determine precision instead of both accuracy and precision.

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ISO cell counting standards have been published to enable better cell counting.

The ISO Cell Counting Standards were published after 5 years of diligent work by participants from 13 countries, indicating the global significance of this work to support the development of cell and gene therapy, as well as regenerative medicine. This process identified several key terms and definitions that can be found in the chart below.

An understanding of the key terminologies from the ISO cell counting standards enables researchers to better consider and evaluate cell counting methods.

TERM	DEFINITION
Cell Count	A discrete number of cells
Direct Cell Counting	Counting method in which one signal is detected for each single event
Differential Cell Count	Number of a subset of cells distinguished from other cell subpopulations by at least one distinct cell attribute
Viable Cells	Defined cells within a sample with the attributes of being alive and healthy enough for the intended use. Viable cells are metabolically active, capable of reproduction, and possess an intact cell membrane
Measured	Quantity intended to be measured. Different measurands can be detected from the same measurement process simultaneously

3 Choose an appropriate cell counting method.

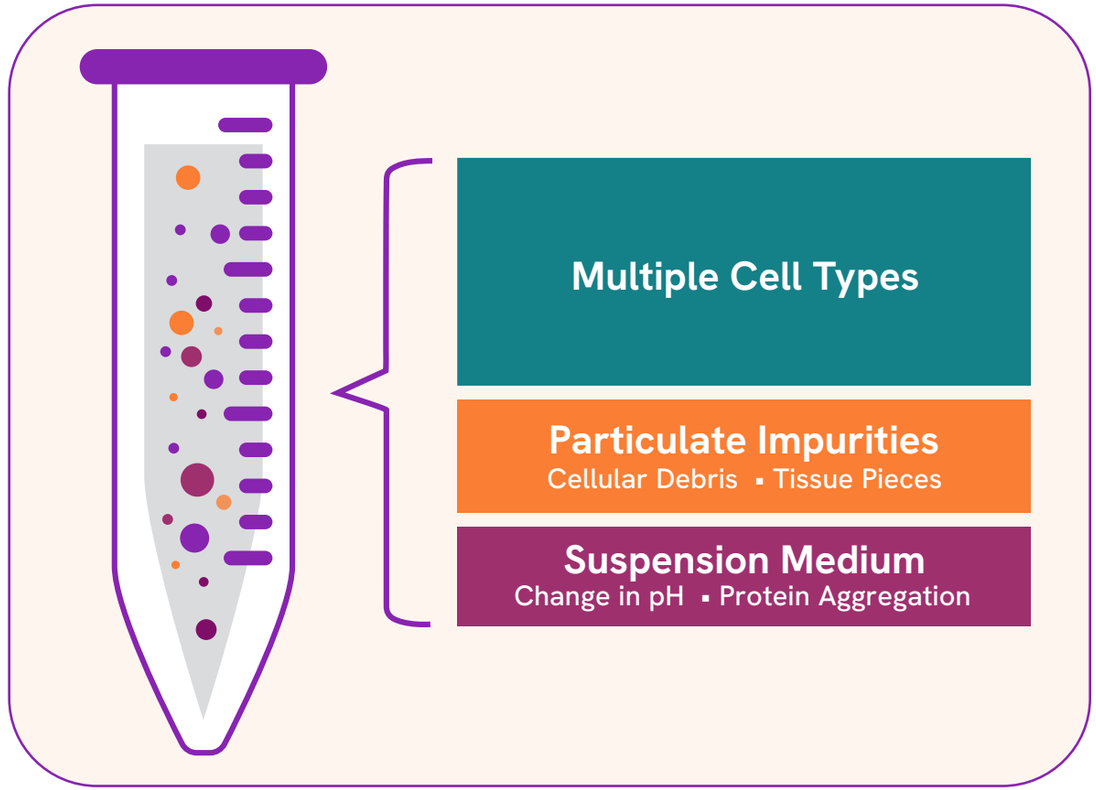
There are many different purposes for which a cell count may be performed. One of the main concepts of the ISO Cell Counting Standard is that the cell counting method should be chosen or optimized according to its "fitness for the intended purpose in line with prearranged requirements for an intended use".[2] In other words, cell counting methods should be fit-for-purpose or tailored to the specific experiment instead of adopting a one-size fits all approach.

Fundamental questions to consider when selecting a cell counting method

- Does the selected cell counting method produce results with sufficient accuracy, precision, and robustness to enable good subsequent decision-making?
- After the cell counting data is obtained, what is the next step in the operation?
- How sensitive are the downstream processes to variation (uncertainty) in the cell count?

4 A critical step: investigating cell sample composition.

Cell samples are frequently contaminated with visible debris and may contain several cell types in addition to the cells of interest (Figure 2). Cells can also stick or clump together and sample composition and appearance can vary during different bioprocesses (e.g., CD34+ purification, CD3 enrichment, PBMC Ficoll). Some of these challenging cell samples may reduce the accuracy of certain cell counting methods or make them impossible to perform. These challenges make understanding the cell sample composition critical to determining a fit-for-purpose cell counting method.



| Figure 2: An example of cell sample composition

5 Choose a cell identification methodology suitable for each sample type and cell type.

Non-fluorescent (colorimetric, Figure 3) and fluorescent (Figure 4) molecular probes are typically used to provide measurements of cell health, viability, and identification. There are several selection criteria related to cell identification methodologies such as biological phenomenon, measurands, probes, and signals. The selected probes can determine if the cell membrane is intact, if the cells are metabolically active, or if apoptosis has been initiated.

Brightfield – Low Viability

Measurand: Live and Dead Cells
Biological Phenomenon: Cell Membrane Permeability
Probe of Choice: Trypan Blue
Signals Detected: Live (refractive light), Dead (blue color)

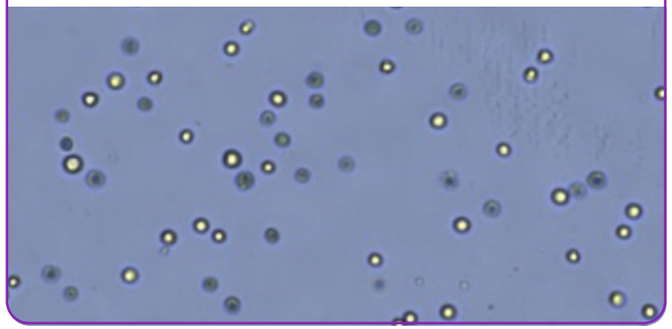


Figure 3: Trypan blue stained CHO-S cells showing live (bright refractive light) and dead (dark blue) cell counting via cell membrane permeability assessment.

Brightfield

Measurand: Live and Dead Cells
Biological Phenomenon:
Cell Membrane Permeability

AO/PI Fluorescence

Probe of Choice: Trypan Blue
Signals Detected: Live (refractive light), Dead (blue color)

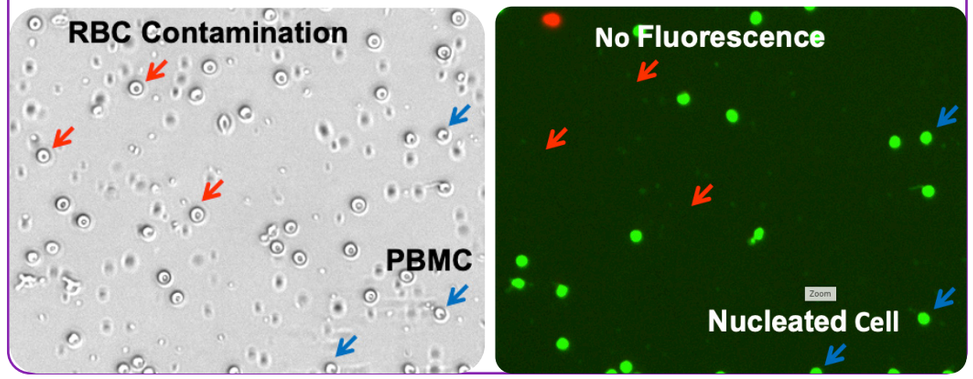


Figure 4: AO/PI stained PBMCs showing live (AO-green FL) and dead (PI-red FL) cell counting via cell membrane permeability assessment.

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A cell counting system includes more than an instrument.

It is important to understand that a cell counting system is not just an instrument (Figure 5). It also includes reagents, consumables, and analysis software, which all play a critical role in generating high-quality results.

Figure 5: Cell counting system reagents and consumables

Parameters to consider when selecting a cell counting system:

- How does the **cell counting system** produce cell counting results?
- How many **cell identification methods** does the instrument have?
- Does the system include the multiple cell identification methods that may be necessary to achieve **fit-for-purpose results** for variable biological samples?
- Does the software provide **specialized and advanced algorithms** to handle variation in cell shape, size, and clumping - particularly when circumstances require challenging brightfield counting?
- Can the instrument use **various reagents** to handle a variety of sample compositions?
- Does the instrument handle a **broad range of cell counts** (not including dilution)?
- What is the **instrument-to-instrument variation**?
- Can the instrument measure both **total cell count** (or viable cell count) and **viability** at the same time, from the same cell sample?
- What is the **measurement precision** of the most important cell count assay for the intended application?
- Can the system provide the necessary **speed and throughput**?
- Is the system part of a larger product family that can facilitate **method transfer** fromdiscovery to development to manufacturing?

7 The quality of results depends upon the entire cell counting process.

The overall quality of cell counting and viability data is determined by the entire cell counting process, not just the cell counting instrument. Any changes to the parameters in any part of the process may affect the cell counting results (Figure 6).

Multiple parameters can generate variability in cell counting assays including the number of samples aliquoted, diluted, mixed, treated, and how long the cell samples are stained. These parameters are related to the entire cell counting process and identifying key parameters in your process can improve your cell counting results.

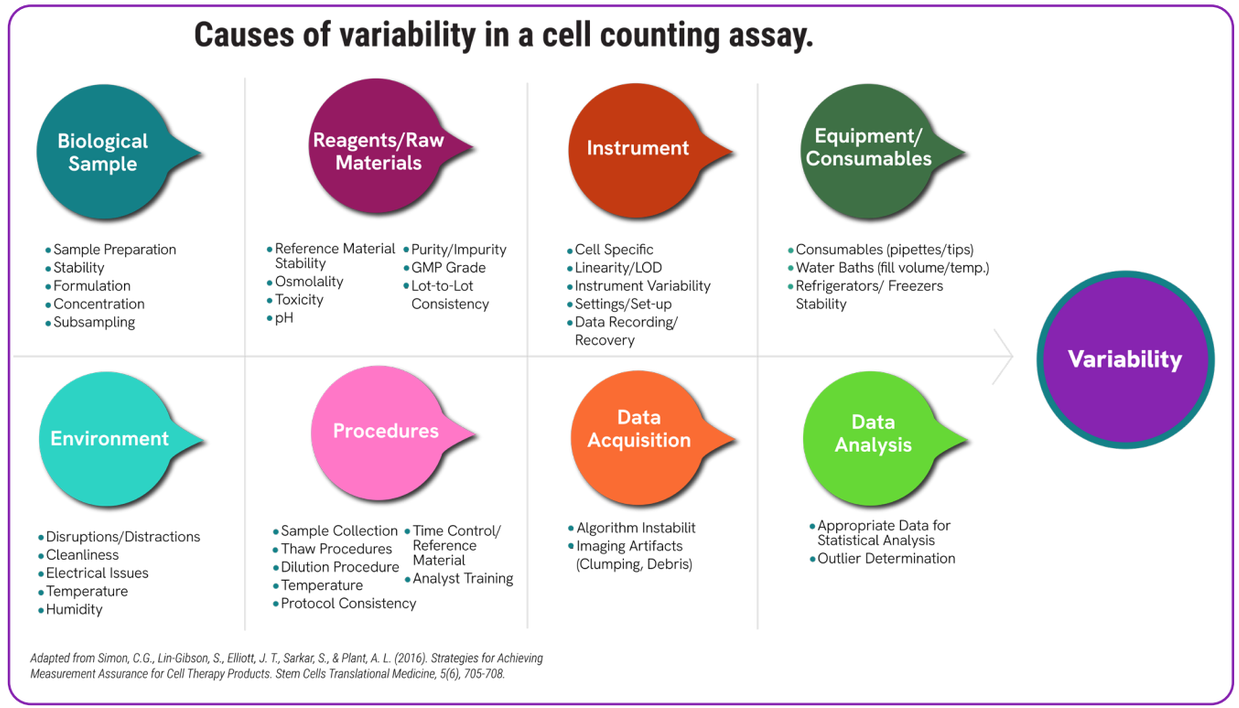

Figure 6: Parameters that may cause variability in a cell counting assay.

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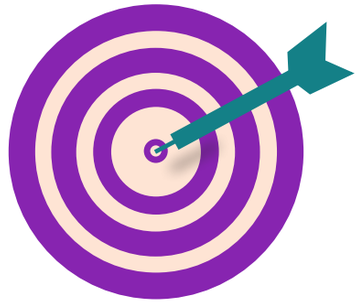
More cells and samples in a properly executed measurement can increase its statistical value.

When performing cell counting measurements, appropriate expectations should be set for statistical variation based on the experimental conditions. Collecting sufficient data improves the quality of cell counting results with three levels of cell counting precision to consider. For example, the coefficient of variation (CV) used to determine precision of a cell counting experiment can be correlated to $\frac{1}{\sqrt{n}}$, where n is the number of cells counted. A cell counting experiment with 100 cells counted at the minimum will still generate a 10% random error even if the sample preparation is perfect.

Repeatability

Measurements of the same sample under similar conditions



Intermediate precision

Measurements taken by different operators, on different days, using different instruments, etc. (representing the normal amount of variation in day-to-day lab operations)

Reproducibility

Measurements made in different laboratories

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Effective strategies have been developed for evaluating and comparing cell counting methods.

Traditionally, cell counting methods are compared using bench-marking experiments between the established and new methods. This is insufficient since the absence of live-cell reference standards makes the traditional accuracy evaluation impossible. While determining which method is more accurate is difficult, it is often useful to measure the bias between two methods using the Bland-Altman methodology.[5,6] The ISO Cell Counting Standard Part 2 is used to evaluate the quality of the cell counting method.[3] The guidance provides protocols including multiple dilutions, replicates, and observations to reduce the effect of noise in proportionality measurements. The results consider quality parameters for linearity (coefficient of determination, R2), precision (coefficient of variation, CV), and proportionality (proportionality index, PI) to determine a fit-for-purpose cell counting method.

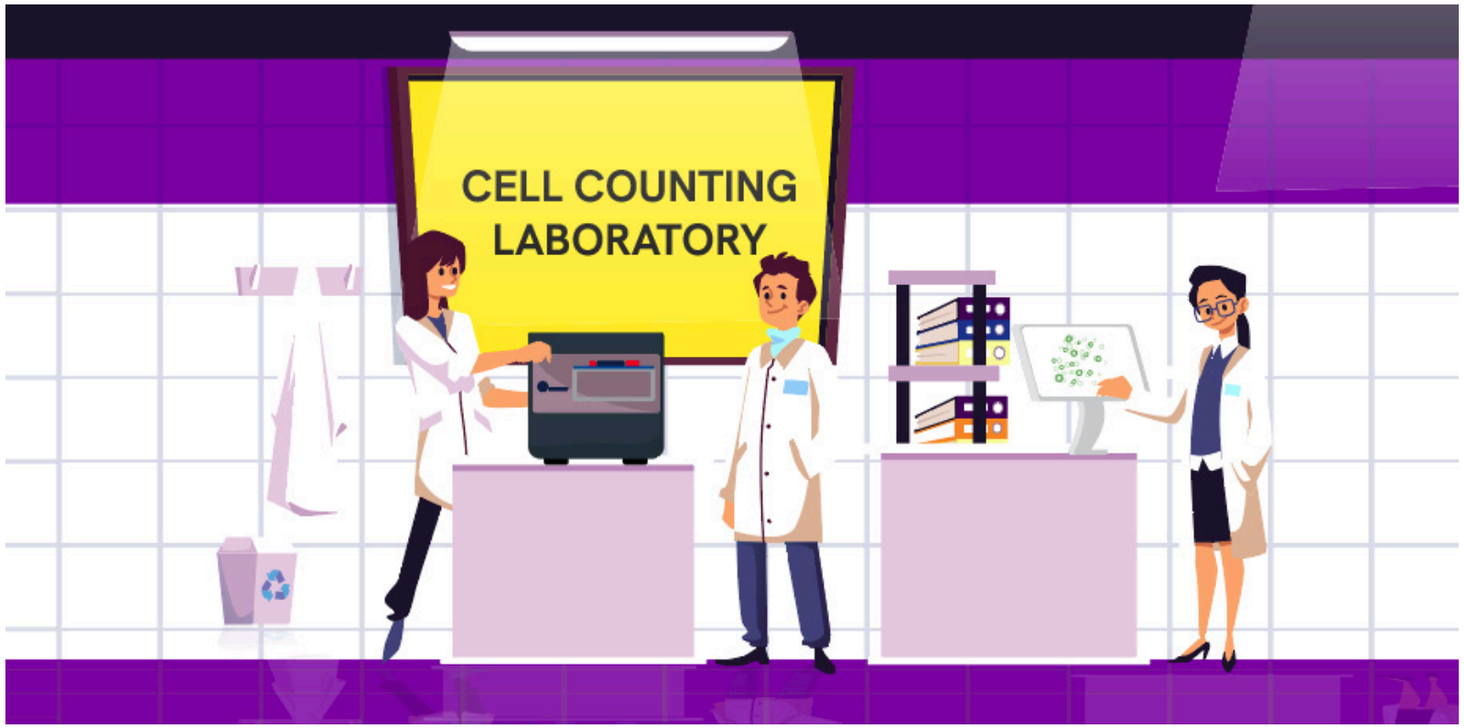
What to consider when comparing cell counting methods

- Don't expect or insist that results from two cell counting methods match exactly
- Don't assume that a method is of higher quality simply because it is already in use
- Even two “perfect” methods will disagree if they depend on different methods of cell identification
- Verify the proportionality of both methods, and determine the relationship between their results for each concentration
- Use Bland-Altman analysis to easily calculate and visualize the difference between methods

10

In-depth and continuous training of operators is essential for long-term success.

It is important to conduct regular training sessions for cell counting operators at all levels because cell counting is a process that will require consistent operation for each step to produce consistent results.



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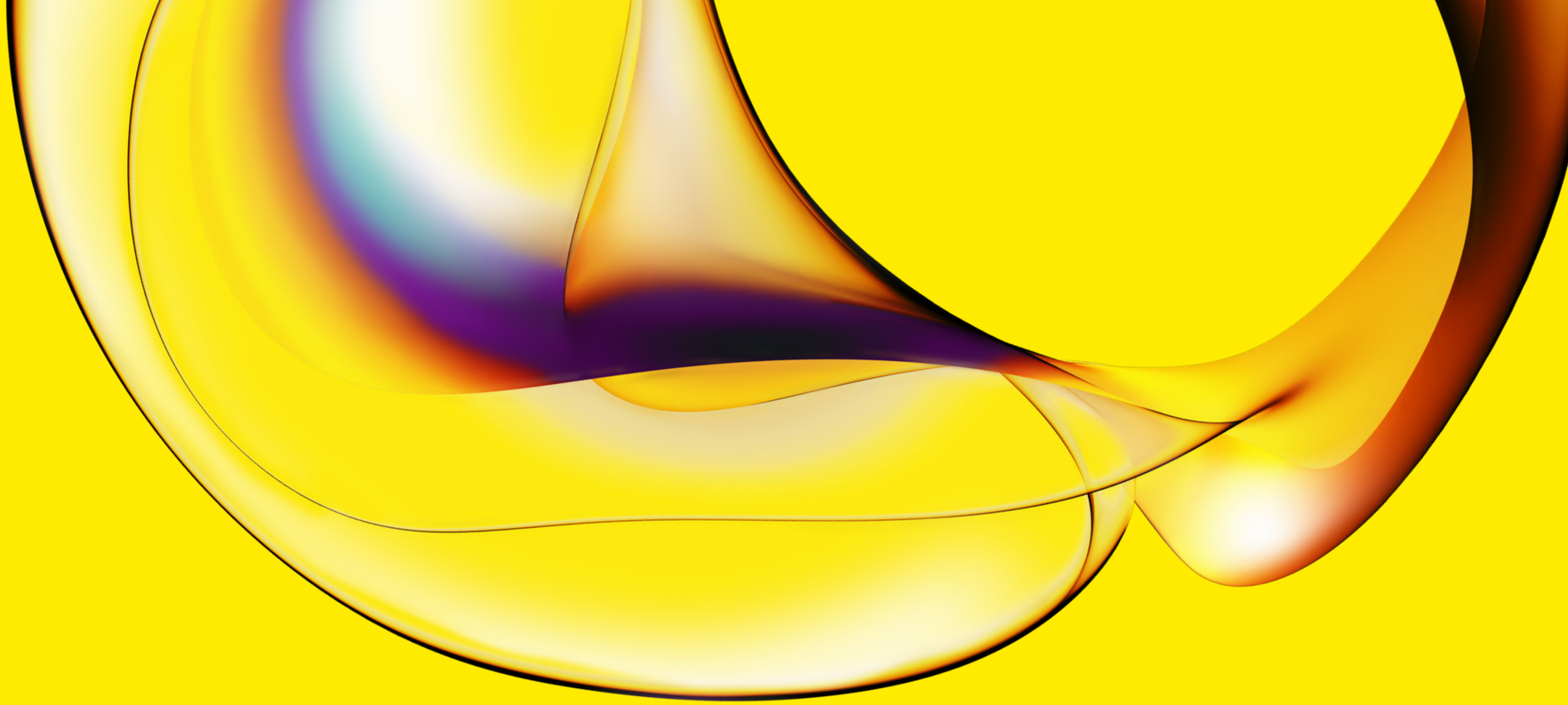

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