Exploring factors impacting and improving cell counting precision

Introduction

Cell counting precision is a critical parameter for determining the overall quality of cell counting results. Many factors contribute to cell counting variability with the potential to impact precision. Any change to the parameters in the entire cell counting process, including sample collection and preparation, cell transfers to counting vessels, imaging, data acquisition and analysis, and output, may affect the cell counting results. Figure 1 shows a comprehensive list of factors that may cause cell counting variability.

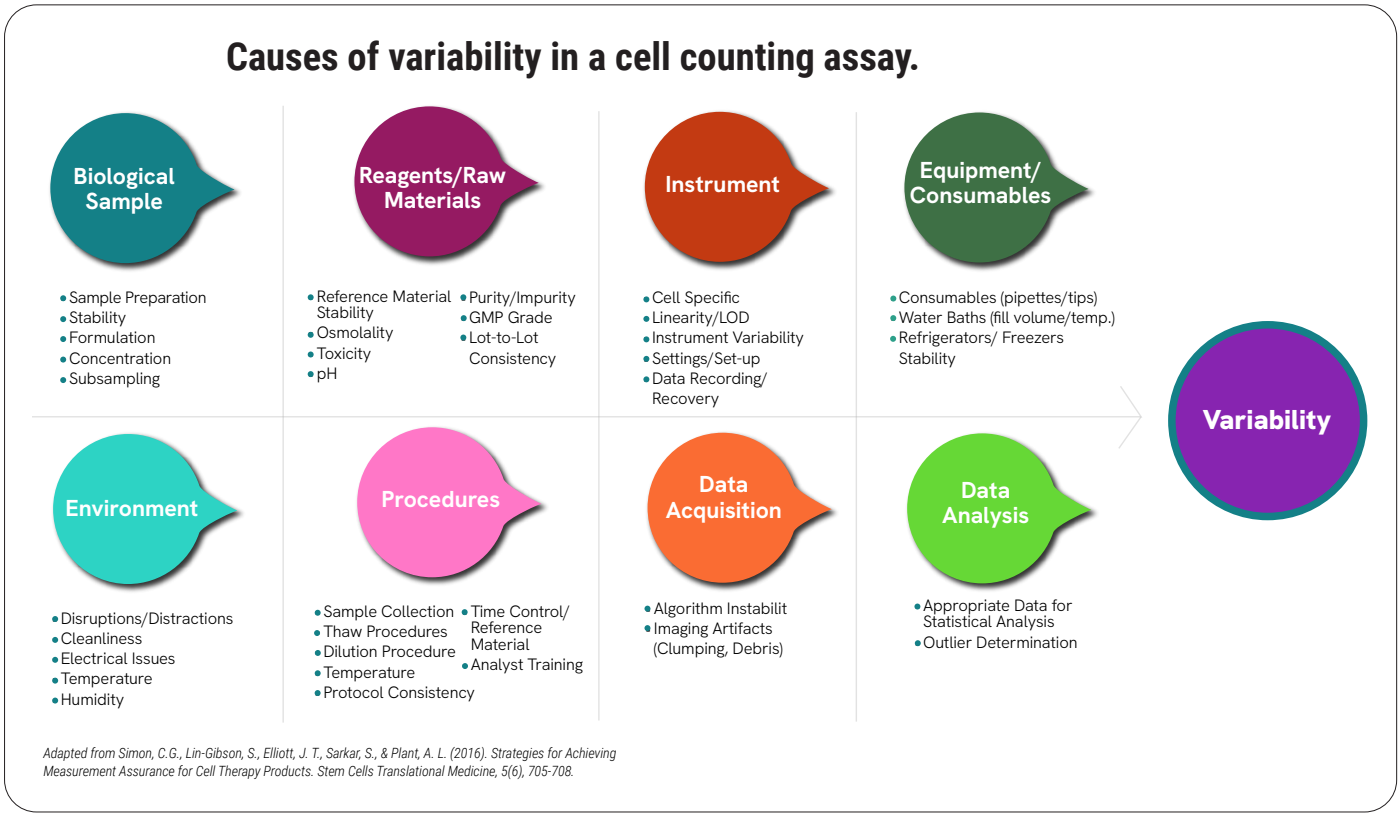

Figure 1: Parameters that may cause variability in a cell counting assay.

In this white paper, we will discuss:

- What precision is and why it is important for cell counting.
- The correlation between precision and Poisson Noise, and strategies to reduce the latter.
- Recommendations on practices and procedures to improve cell counting precision.
- Three cases that demonstrate how parameters in the cell counting process can affect cell counting precision.

Precision versus accuracy

Understanding the importance of cell counting precision begins with understanding what precision is. The definition provided by the ISO Cell Counting Standard Part 1 stated that precision is the "closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions" (ISO 2018, Huang, Bell et al. 2021).

The graph in Figure 2 demonstrates the relationship between precision and accuracy, where the red dots indicate the measurement values that may land somewhere on a target. The closer to the center of the target, the more accurate the data set is, whereas the precision is indicated by the tightness of the red dots. Since there are no live cell reference standards in cell counting, we do not have a target to compare to (ISO 2018, Huang, Bell et al. 2021), instead, researchers need to utilize an orthogonal counting method such as a hemacytometer (Figure 3).

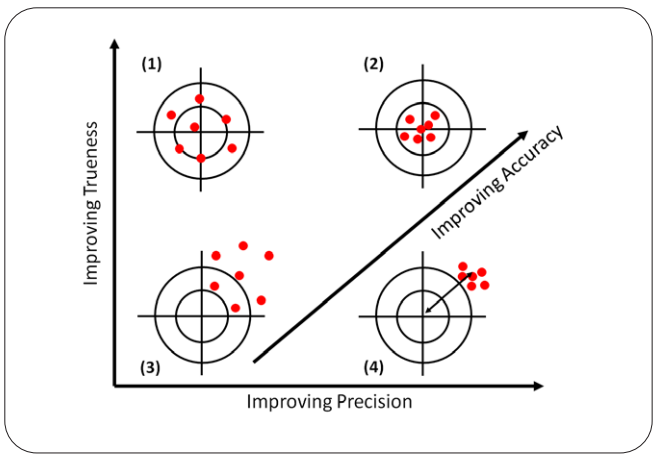

Figure 2: The relationship between accuracy and precision.

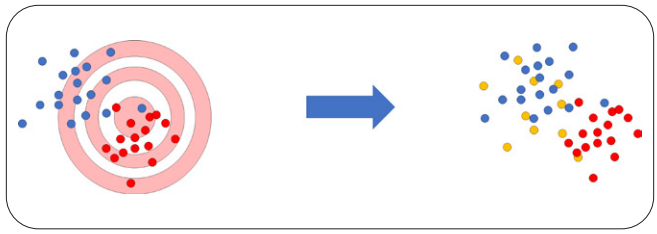

Figure 3: In cell counting, there is no live cell reference standard which means that the target does not exist. Orthogonal methods are recommended for comparison.

Cell counting precision

There are three different levels of precision (Bell, Huang et al. 2021): measurement repeatability, intermediate measurement precision, and measurement reproducibility with increasing uncertainty (Figure 4). Measurement repeatability is repeated measurements of the same or similar samples under similar conditions. Intermediate measurement precision is repeated measurements with different instruments, operators, days, lots, or a combination of them. Measurement reproducibility is repeated measurements in different laboratories.

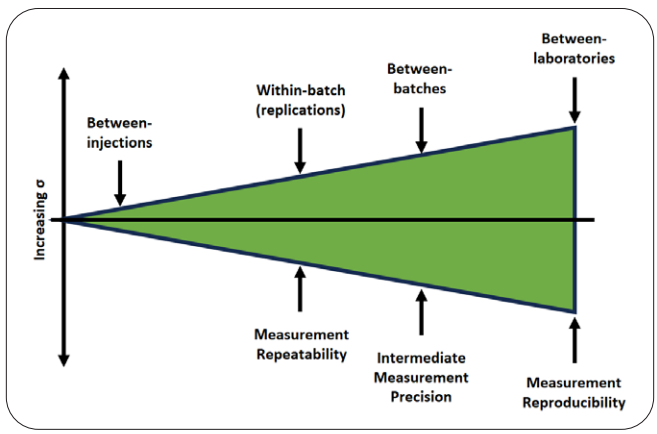

Figure 4: Different levels of precision with respect to σ (standard deviation).

Cell counting precision is essentially the measurement uncertainty generated by the sources of variability. For example, repeated measurements will produce a distribution of values instead of a single value. This measurement uncertainty is often described as the "standard measurement uncertainty" (standard deviation) or "relative standard measurement uncertainty" (coefficient of variation or CV), where $\%CV = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$, representing cell counting precision.

Poisson noise

Each cell counting methodology has unique sources of variability that can reduce cell counting precision. One consistent source of variability is the Poisson Noise or Shot Noise related to the random error during cell counting. In general, a cell counting method cannot be made more precise than its Poisson Noise, which is a basic form of uncertainty associated with counting discrete events or objects like cells.

In Figure 5, assume the red cells are randomly distributed in a box as shown. By sampling multiple locations within the box, as shown with the green circles, there is inherent variation in the number of cells sampled. A lower number of objects captured can reduce precision. Even if the rest of the cell counting process is perfect, this variation or Poisson Noise still exists. The relative standard uncertainty (CV) due to Poisson Noise can be correlated to ~ $\frac{1}{\sqrt{n}}$, where n is the average number of counted cells per sample. For example, if a researcher counted 10, 100, and 1000 cells, the CV would be approximately 32, 10, and 3.2%, respectively. Typically, manual cell counting using a hemacytometer counts around 100 cells, thus 10% of CV would be a precision level commonly observed. Ultimately, increasing the number of cells counted per sample will increase precision.

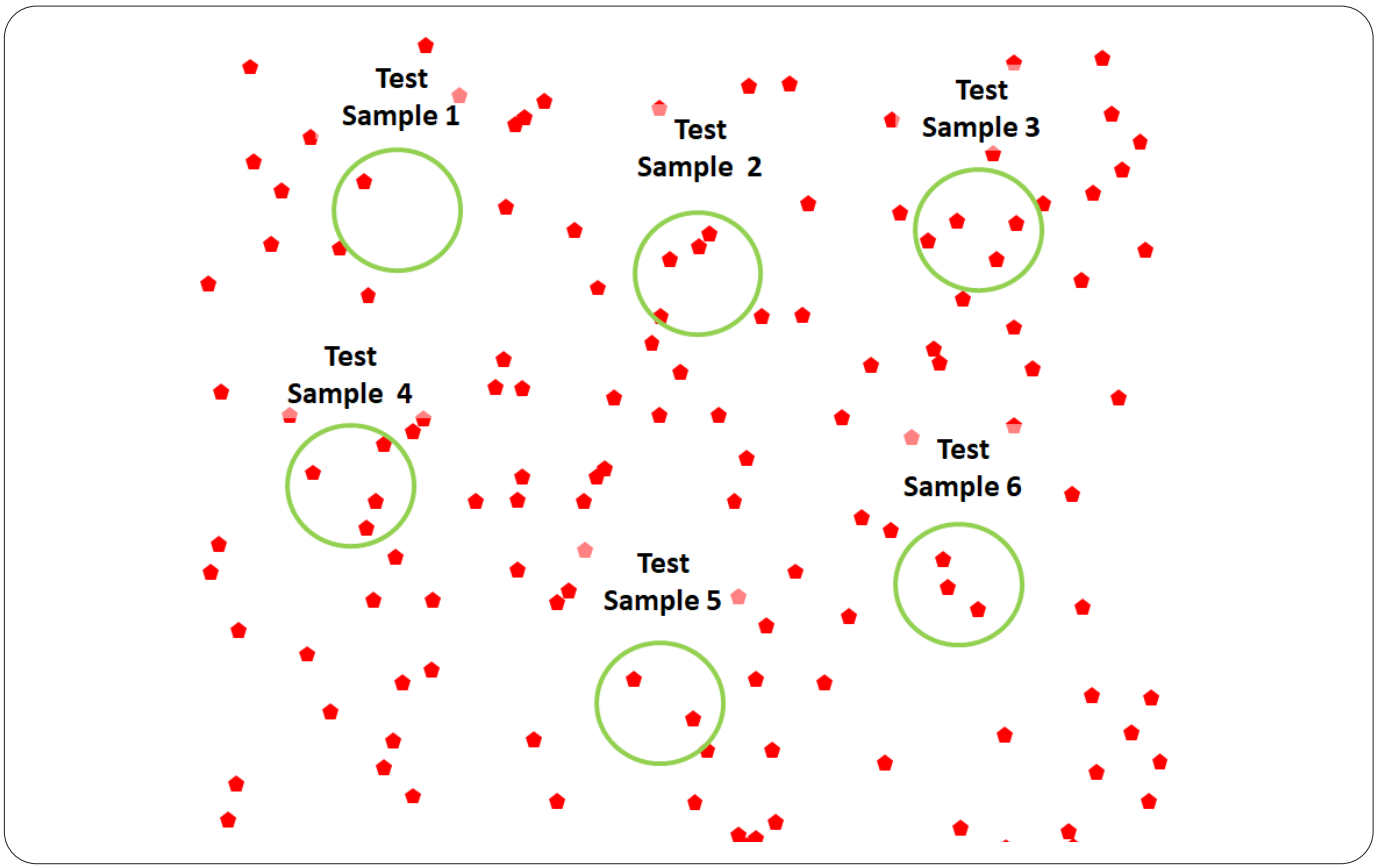

Figure 5: Example of Poisson Noise in cell counting. Sampling multiple areas, within the green circles, shows inherent variation in the number of cells.

Perfect cell counts from a well-mixed cell suspension will follow a Poisson distribution and essentially becomes a Normal distribution when more than 30 cells are counted (Figure 6A). In statistical analysis, the standard deviation (σ) in a Normal distribution is used to describe the probability coverage, where ±1, 2, or 3σ correlates to the probability that 68.3, 95.5, or 99.7% of counting results are located within said range. The width of the distribution depicts the variation of the cell counting method, where wider indicates lower precision. Counting more cells per measurement results in a relatively narrower distribution indicating higher precision (Figure 6B)

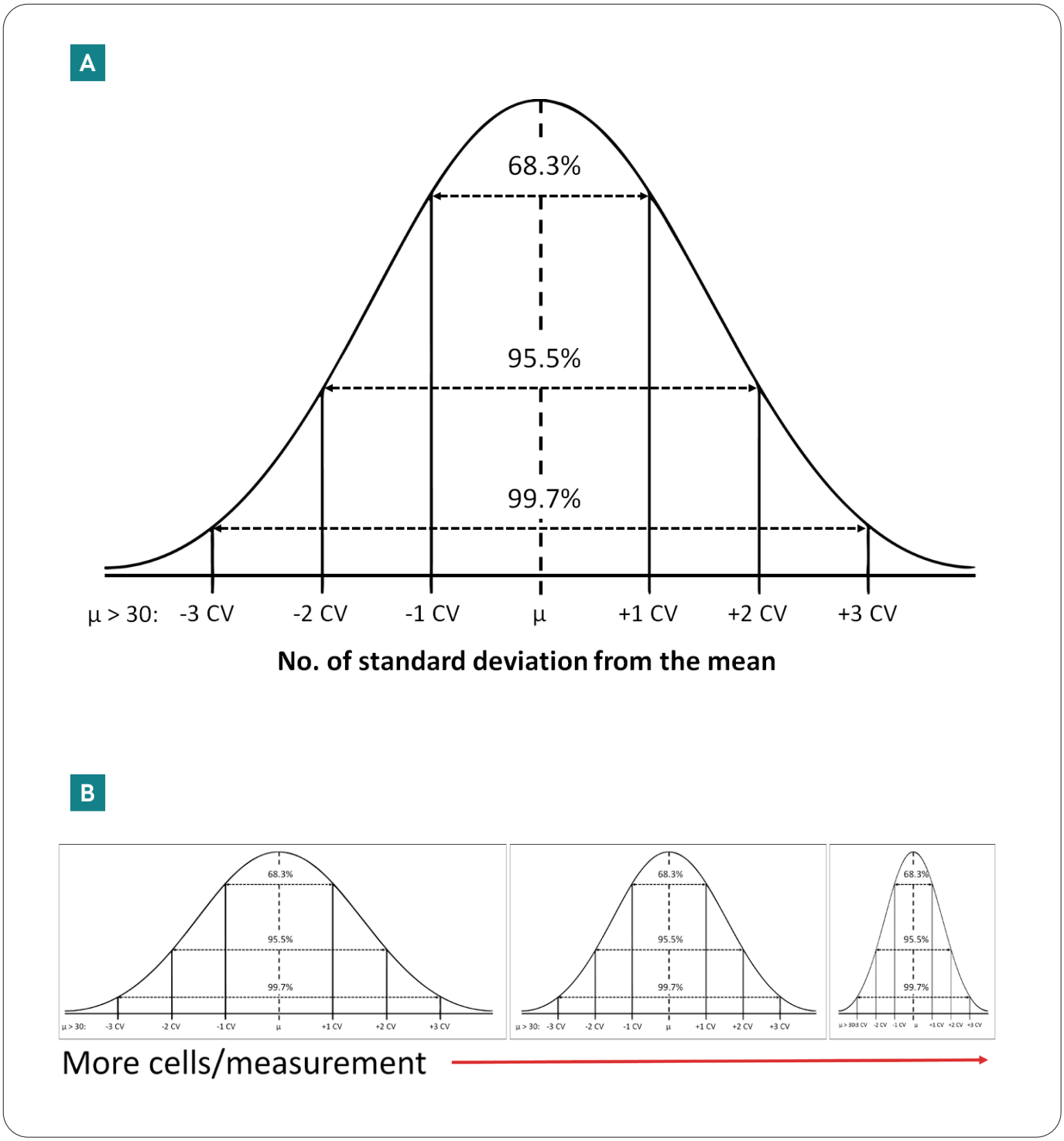

Figure 6: Example of (A) Poisson Distribution demonstrating the probability plot in respect to standard deviation. (B) As the number of cells counted increases, the distribution narrows.

Evaluation uncertainty of a cell counting method

Cell counting variability is a combination of Poisson Noise and other parameters within the entire cell counting process. We may evaluate cell counting variability with two types of methods. Type A evaluation focuses on the statistical analysis of measured quantity values obtained under defined measurement conditions. Type B evaluation focuses on any other type of measurement evaluation.

Type A evaluation – a simple method

The simple method for evaluating cell counting uncertainty is to assess several cell concentrations in the low, middle, and high end for the target cell types. The researchers should use the current or intended cell counting methods to count the cell samples at 30 or more replicates per concentration, and then calculate the mean (μ), standard deviation (σ), and $CV\% = \frac{\sigma}{\mu} \times 100\%$ for each concentration.

Type A evaluation – ISO Cell Counting Standard Part 2 method

Under the guidance of ISO Cell Counting Standard Part 2, researchers should test a range of independently prepared cell concentrations that is "fit-for-purpose", where multiple replicate samples and measurements are generated for each concentration (ISO 2019). As a result, the precision of the cell counting method is determined by calculating the pooled CV% from the data set.

To determine the pooled CV%, researchers will need to calculate the mean (μ) and variance (VAR or σ^2) for 1st, 2nd, 3rd... replicate from n_1, n_2, n_3, ... respective observations. Next, the weighted mean from all replicates is calculated using the equation $\overline{\mu} = \frac{n_1 m_1 + n_2 m_2 + \cdots}{n_1 + n_2 + \cdots}$ and the pooled variation from all replicates is calculated using the equation $\overline{\sigma^2} = \frac{(n_1-1)\sigma_1^2 + (n_2-1)\sigma_2^2 + \cdots}{(n_1-1)+(n_2-1)+\cdots}$. Finally, use the equation $CV\% = \frac{\sqrt{\overline{\sigma^2}}}{\overline{m}} \times 100\%$ to calculate the final pooled CV from the entire data set.

Type B evaluation

Researchers may obtain the precision of a cell counting method through published sources, a calibration certificate, or a certificate of analysis. In addition, precision can also be obtained from predicted values from a statistical model. Furthermore, they can rely on empirical evidence.

Expected precision from any cell counting methods

Revvity has developed a cell counting precision prediction app to predict the range of CVs expected for a given cell counting experiment. The software application allows users to enter values for the number of replicates per experiment and the number of counted events per count, which is determined by dilution factor and concentration. The prediction model can generate the expected range of CVs from an experiment as shown in Figure 7, where the red lines indicate the upper and lower quantiles of CV% or the 95% confidence interval. A perfect cell counting process follows only the center line (Poisson Noise) correlated to $\frac{1}{\sqrt{n}}$.

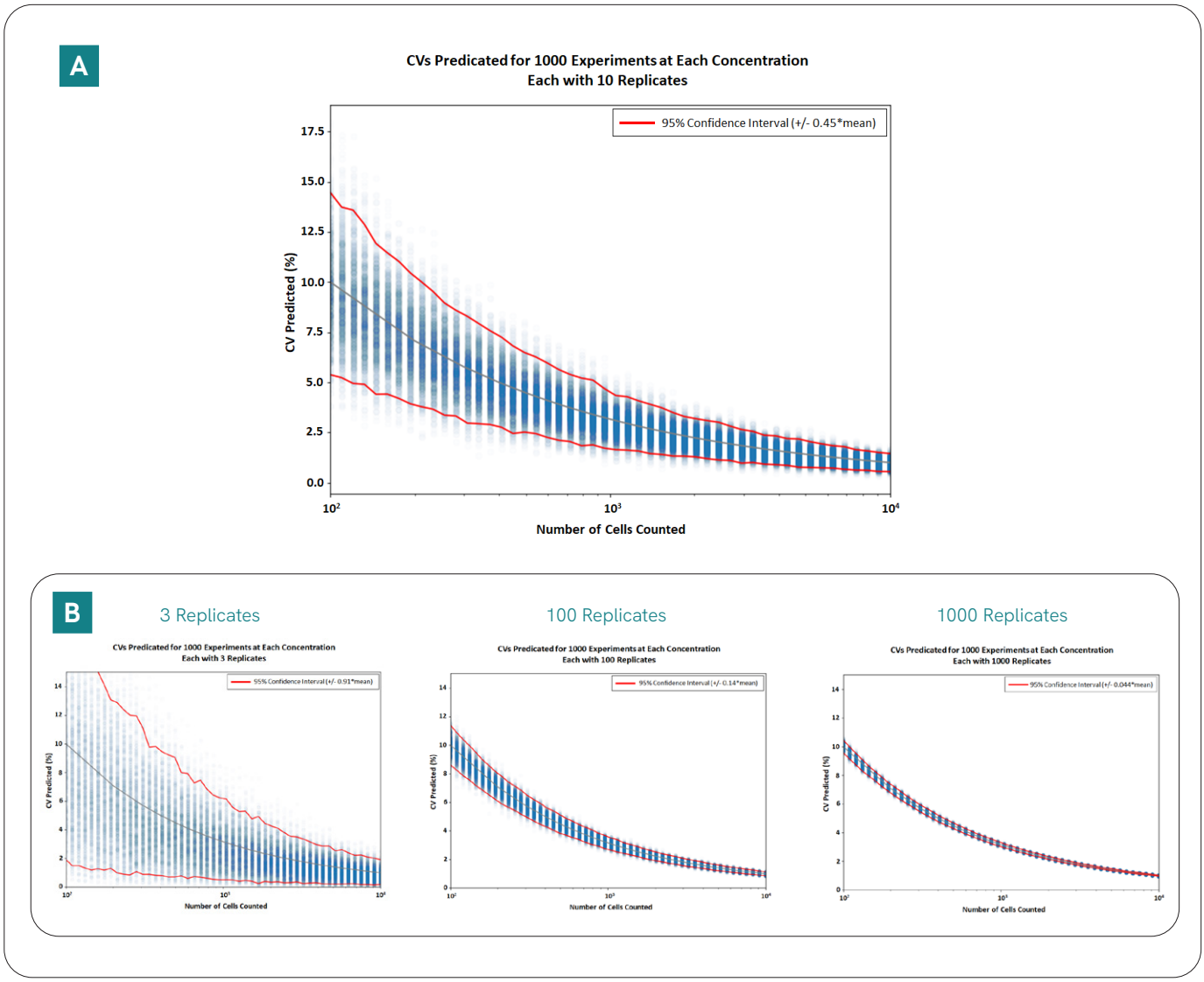

Figure 7: Revvity's precision prediction app used to determine Poisson Distribution for cell counting experiments. (A) Example prediction of 10 replicates for 1000 experiments performed. (B) Increasing replicates improves the accuracy of the prediction.

An example is shown in Figure 7. There are three prediction models generated with replicate samples of 3, 100, and 1000 with 1000 experiments performed. As a result, the more replicates per experiment, the more accurate the prediction of the experimental CVs, meaning a clearer picture of the real variation within a cell counting method. One might ask, how many replicates should be done? It truly depends on how close to the true CV is required.

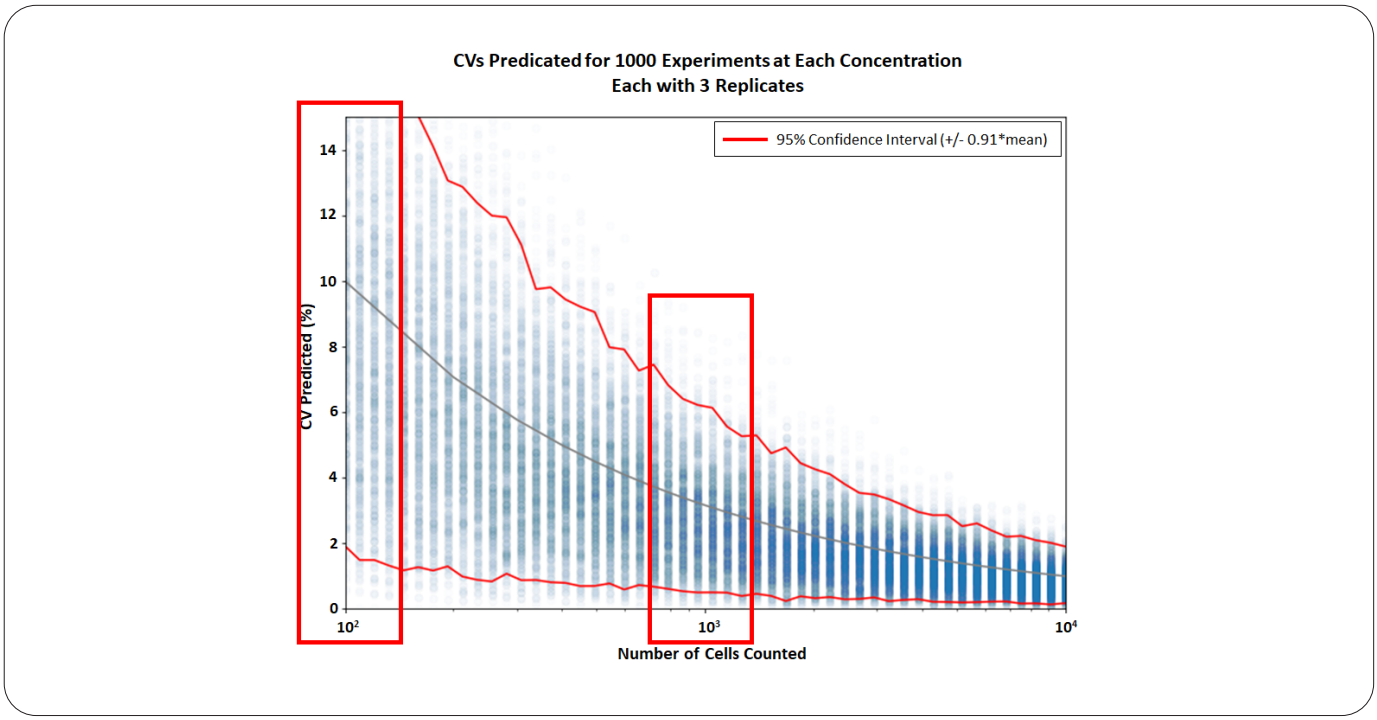

Figure 8: Typical cell counting precision prediction at 3 replicates.

Less than three replicates are usually measured in a cell counting experiment. If 100 cells are counted at 3 replicates, the CV can range from 2 to more than 14% with an average of 10%, which can be correlated to manual counting with a hemacytometer (Figure 8). With the current advancements, if 1000 cells are counted at 3 replicates, the CV can range from 1 to 6.5% with an average of 3.5%, which can be correlated to automated cell counters (Figure 8).

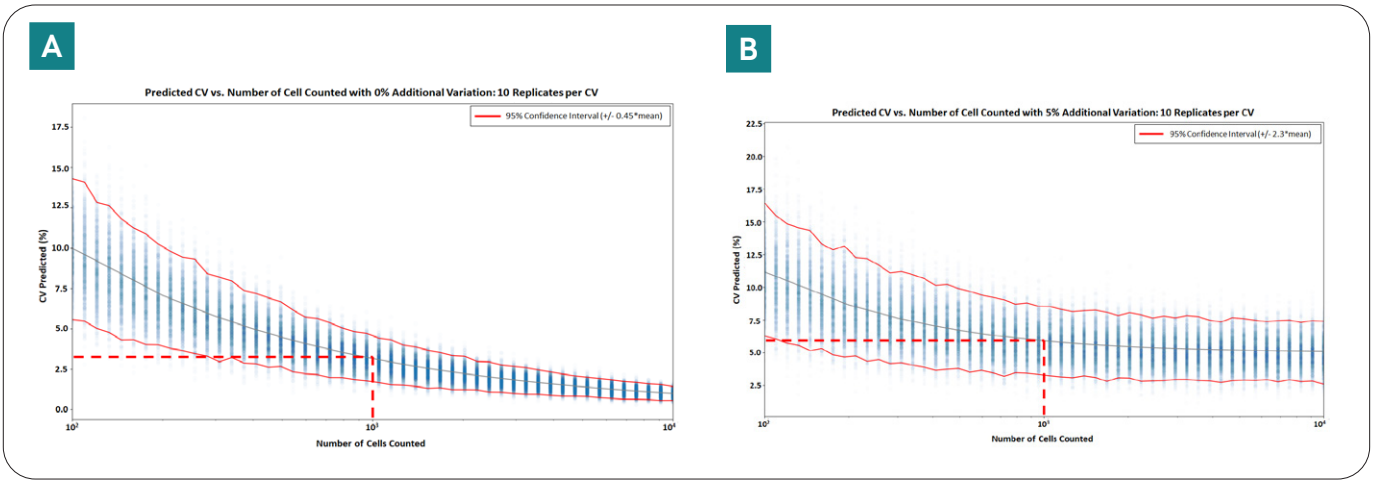

Figure 9: Example prediction of cell counting precision. (A) Predicted CV for 10 replicates per experiment with a "perfect" cell counting process. (B) Predicted CV for 10 replicates per experiment with additional 5% variation from cell counting process.

Other sources of variation

For a cell counting experiment with 10 replicates and a "perfect" cell counting process, the predicted CV is approximately 3% at 1000 cells counted. Realistically, we can add in a 5% cell counting process variation, which brings the average CV to approximately 6% and increases the range of the CV (Figure 9).

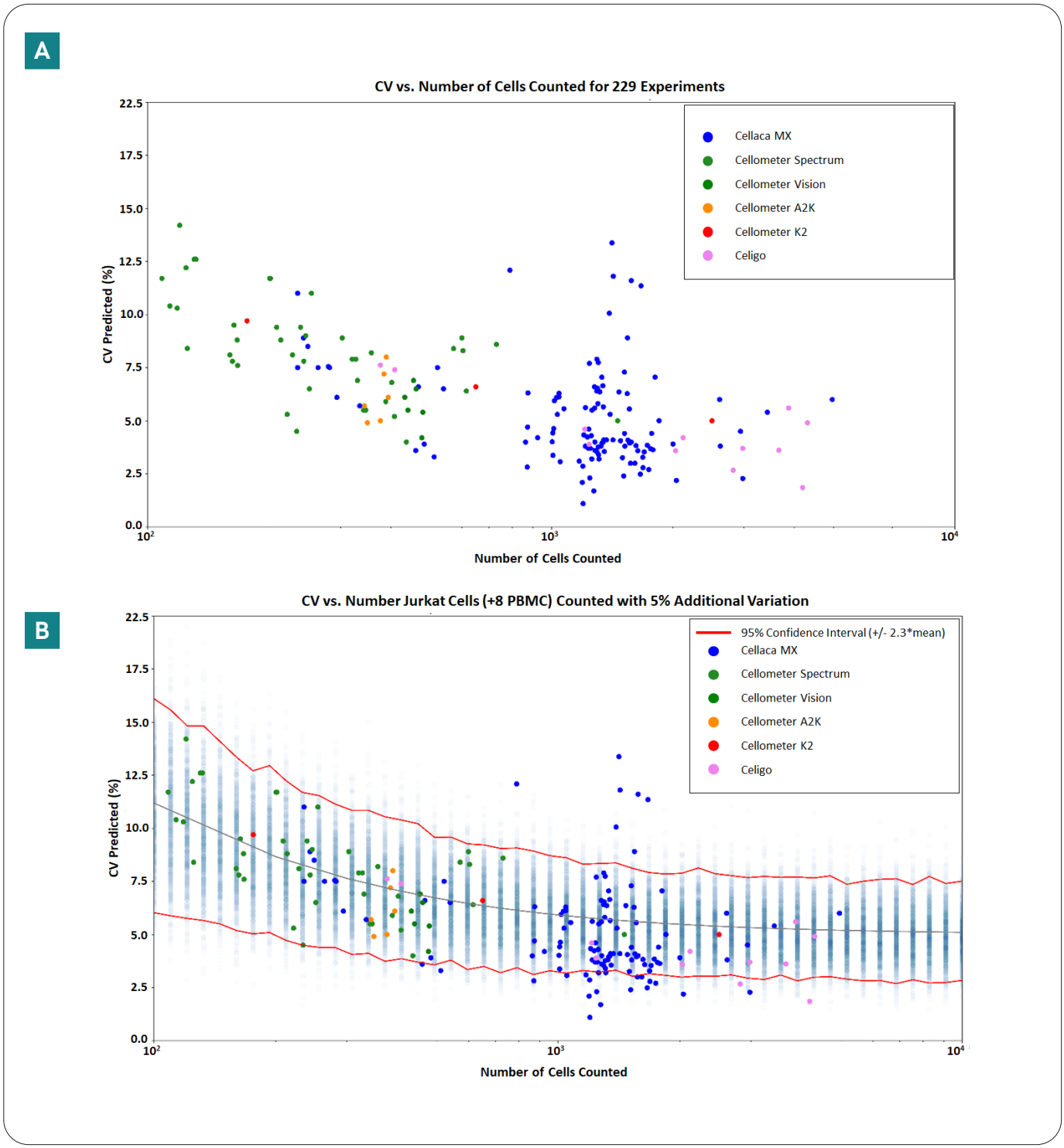

Figure 10: Combined empirical cell counting CV results showing a combination of Poisson Noise and variation from cell counting process. (A) Empirical data of 229 experiments with replicates per experiment ranging from 4 to 24. (B) Predicted CV overlaid with empirical CV data.

We have compiled empirical CV data from 229 experiments with replicates per experiment ranging from 4 – 24, which followed the CV prediction app closely with added 5% cell counting process variation (Figure 10). Some outlying CV data was generated above the predicted maximum CV, which we hypothesize are contributed by other variations during the cell counting process such as loss of focus during image acquisition (Figure 11). Other potential sources of variation are shown in Table 1.

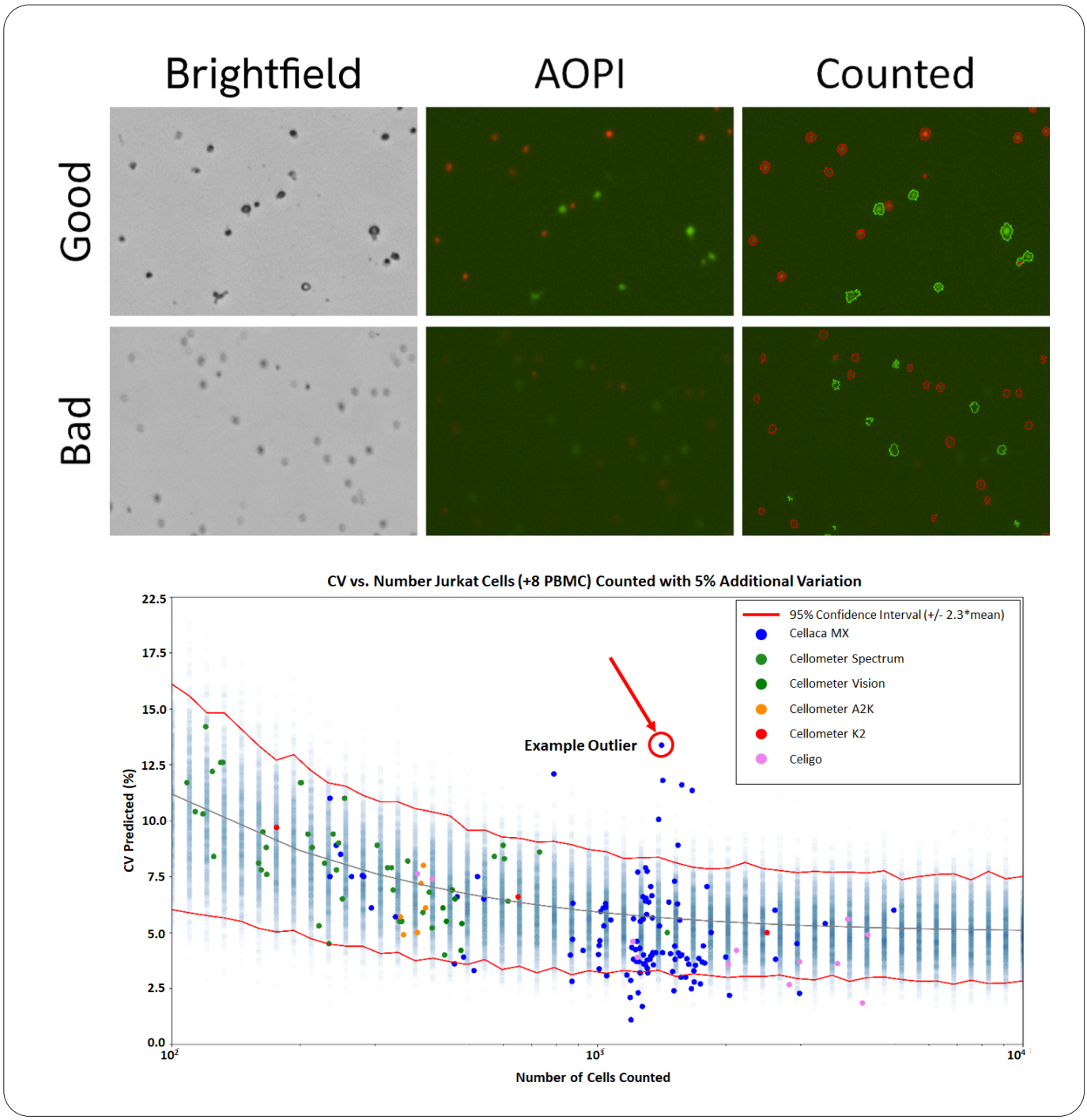

Figure 11: Visible outlier caused by focusing variability.

Table 1: Potential sources of variation.

Source of Variation	Reason
Biological sample	Stability of the cell sample
Reagent/Raw materials	Stains and dyes interact with cells to cause nonspecific counting
	Stability of stains and dyes
Instrument	Counting range of the instrument
Equipment/Consumables	Variation in consumables
	Variation in pipettors
Environment	Change in temperature, humidity, pH, etc. causing variation in cells, reagents, raw materials, consumables, instruments, etc.
Procedures	Sampling, mixing, and diluting can cause variations dependent on the operators
Data acquisition	Focus adjustment
	Not the exact same sample acquired each time
Data analysis	Image analysis algorithms

We have previously investigated key parameters during the cell counting process: pipetting/sample preparation, slide handling, instrument acquisition, and software algorithm. The sources of variation were determined using Cellometer K2 with polystyrene beads (CCBM) shown in Figure 12 . The results showed that sample preparation in the cell counting process generated the highest variation, as expected.

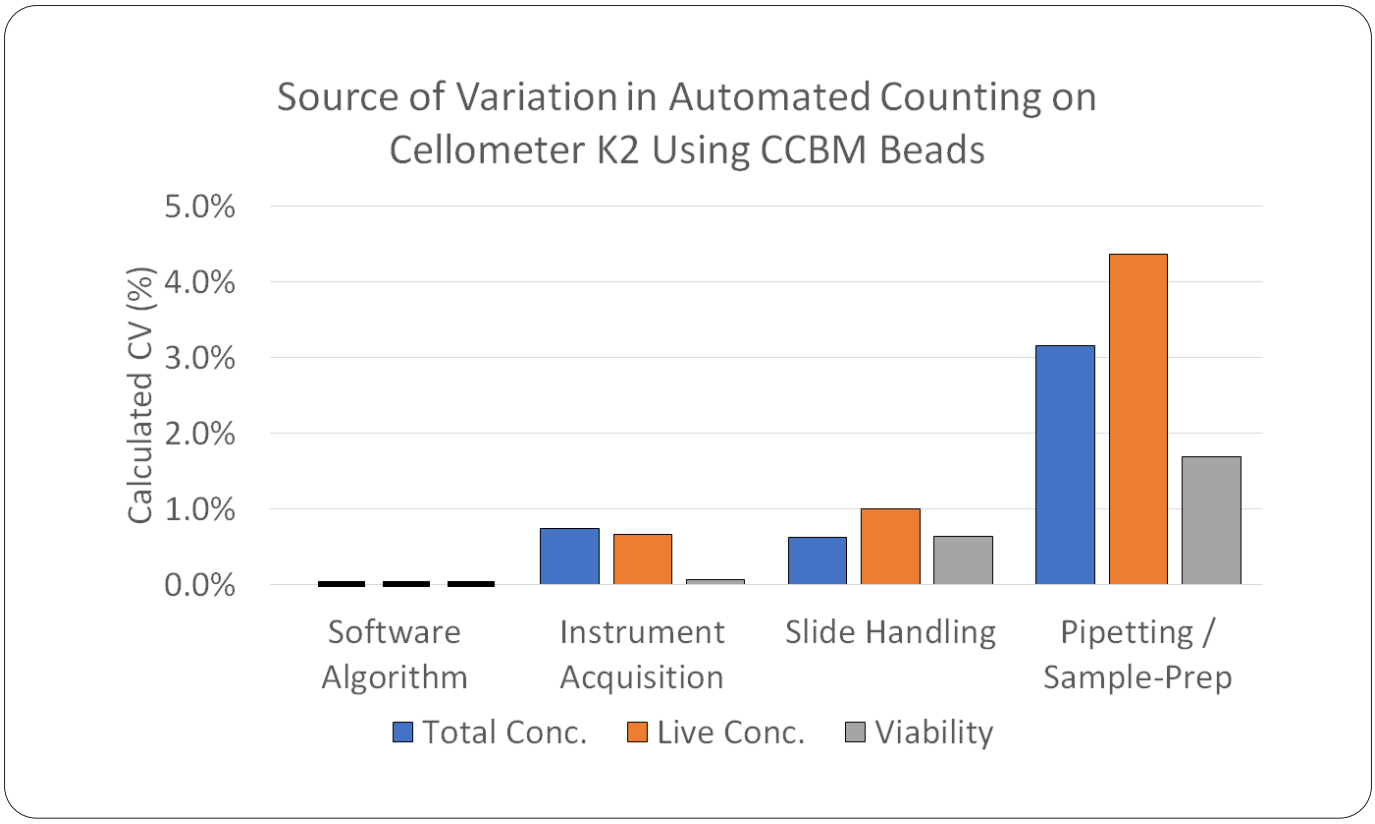

Figure 12: Source of variation in automated counting on the Cellometer K2 cell counter using Cellometer Check Bead Solution.

Recommendations for reducing Poisson noise

The key to minimizing Poisson Noise is to count more cells by measuring a greater volume or by performing several measurements to average together for more volume (Figure 13). This can be achieved by understanding the correct dilution to reach the suggested concentration range of the cell counting method and potentially using a cell counting method with larger counted volumes per measurement (Lin-Gibson, Sarkar et al. 2016). Empirically, we have determined that counting more than 400 cells per measurement can generate approximately 5% CV, given that the cell counting process was performed appropriately.

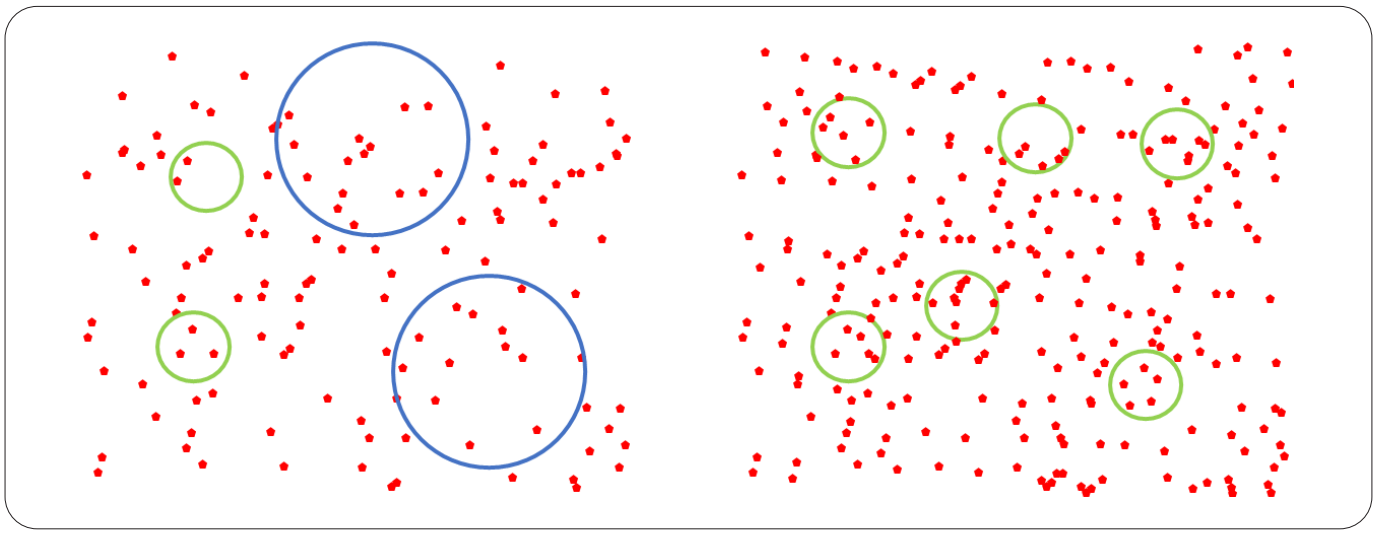

Figure 13: Increasing the volume or concentration improves precision.

Researchers should conduct best practices during the cell counting process to increase operation assurance. For example, cell samples should be carefully prepared and pipetted to minimize large cell counting variation due to human errors. If possible, the number of replicates per sample should be increased to increase the assurance of counting results.

Finally, it is important to understand the entire cell counting process for individual samples and identify all of the parameters that may introduce cell counting variation. The different parameters must then be investigated to understand the expected variation in addition to the Poisson Noise.

Case studies

Here we present three case studies relating to specific parameters that affect cell counting precision.

Case Study 1 – Procedures – Cell settling time (Sample preparation)

We compared the cell concentrations at different cell settling times after vortexing to evaluate how much concentration variation would be introduced. First, 20 µL of Jurkat cell sample at approximately 2 x 10^6 cells/mL was loaded into cell counting chambers CHT4-SD100 (Revvity, Lawrence, MA) at n = 6. The procedure was then repeated for Jurkat cells that were allowed to settle at 0, 1, 5, and 20 min.

The results showed that Jurkat cell concentrations decreased significantly with increasing cell settling time, which introduced significant concentration bias. As a result, the cell counting CV% increased from 2.0 to 25.5% (Figure 14).

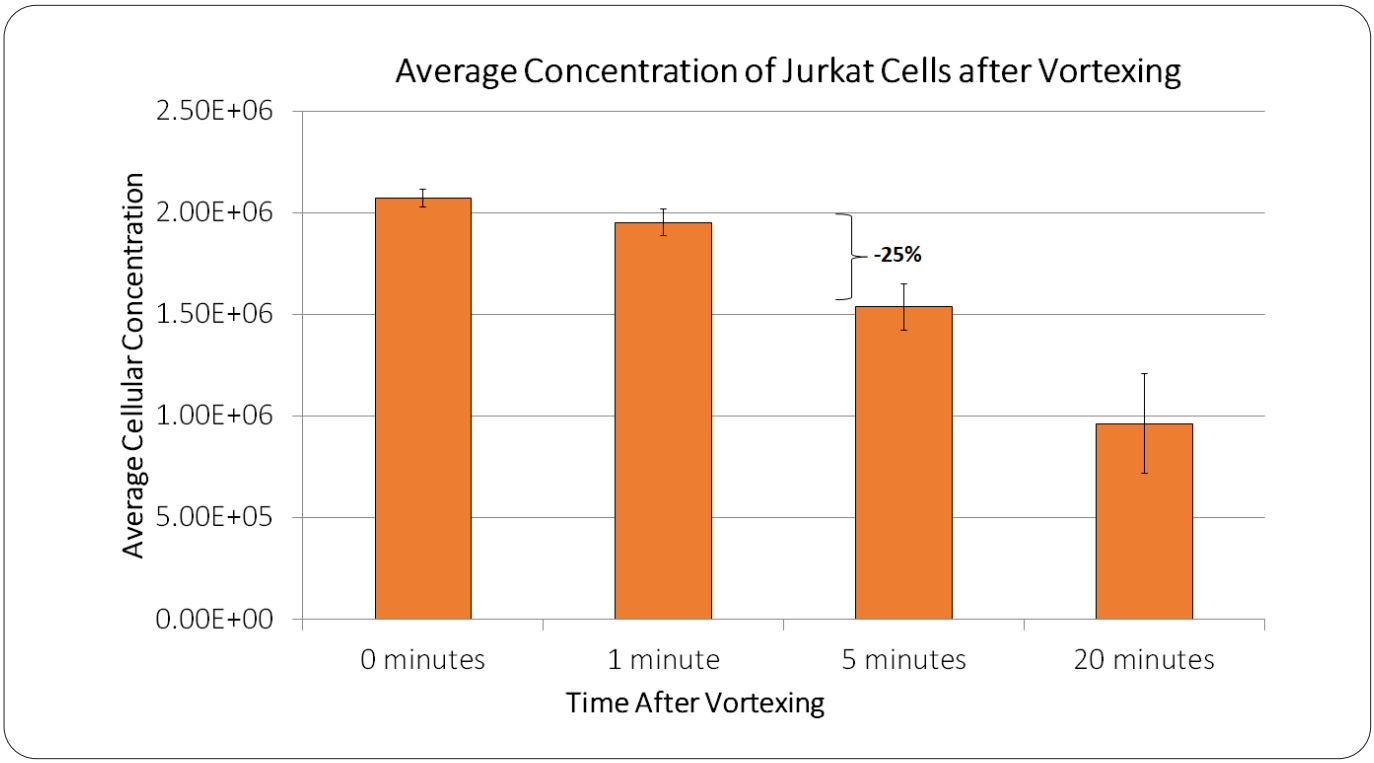

Figure 14: Increasing cell settling time after vortexing introduces significant concentration bias.

Case Study 2 – Procedures – Extra dilution step (Sample preparation)

We compared the effects on cell counting precision when cells are diluted between 2 and 10X. First, a Jurkat cell sample at approximately 5 x 10^6 cells/mL was stained 1:1 with acridine orange and propidium iodide (AO/PI) and mixed uniformly to load into cell counting chambers at n = 12. Next, the same Jurkat cell sample was diluted in media 1:5, stained 1:1 with AO/PI, and loaded into cell counting chambers at n = 12, resulting in a final dilution of 10X. The results showed that with a larger dilution fewer cells were counted, as expected, as well as an increased CV% for total and live cell concentrations (Table 2).

Table 2: An extra dilution step can increase CV% and reduce assay precision

Operation protocol comparison									
DFactor	N Obs	**Count Total**	Conc. Total	**Total CV**	Live Count	Live	**Live CV**	Viability	Viability CV
2	12	1458	4.13E+06	4.9%	1324.83	3.75E+06	5.2%	90.9	1.1%
10	12	321	4.54E+06	7.9%	296.92	4.21E+06	7.5%	92.68	1.8%

Case Study 3 – Data acquisition – Increase the number of captured images (measured cell volume)

We compared the CV% amongst varying numbers of images acquired from the same cell sample. A Jurkat cell sample was prepared at approximately 1.2 x 10^6 cells/mL and stained with AO/PI. The stained cell sample was mixed and loaded into cell counting chambers at n = 12, where 2, 4, and 8 images were acquired. The results showed that as the number of images increased (volume increased), the CV% decreased from 7.7 to 4.7% for total and live cell concentration (Figure 15).

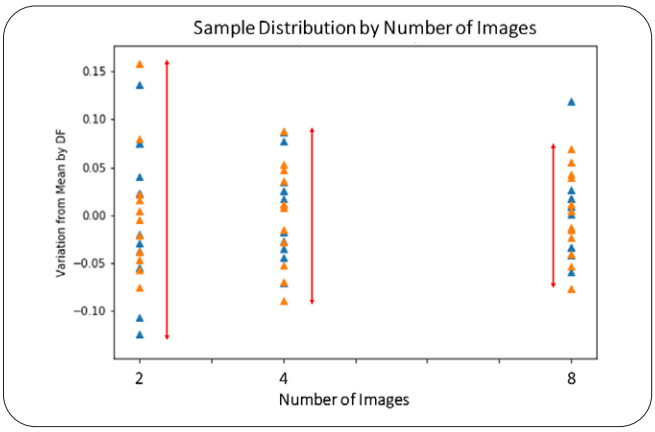

Figure 15: Increasing the number of images increases the number of total cell counts, thus improving the system precision (CV).

Conclusion

In conclusion, measurement uncertainty in cell counting is determined by the entire cell counting process, not just the cell counting instrument. Any change of parameters in the entire process may affect the cell counting results. The causes and effects shown in figure 1 allows investigations into specific parameters that may impact cell counting variability. We recommend three practices to minimize cell counting variability:

1. Increase the number of counted events per measurement to reduce Poisson Noise (> 400 is best).
2. Conduct best practices during the cell counting process to increase the operation assurance.
3. Understand your entire cell counting process.

References

Bell, J., et al. (2021). "Characterization of a novel high-throughput, high-speed and high-precision plate-based image cytometric cell counting method." Cell and Gene Therapy Insights 7(4): 427-447.

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Lin-Gibson, S., et al. (2016). "Understanding and managing sources of variability in cell measurements." Cell & Gene Therapy Insights 2(6): 663-673.

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