

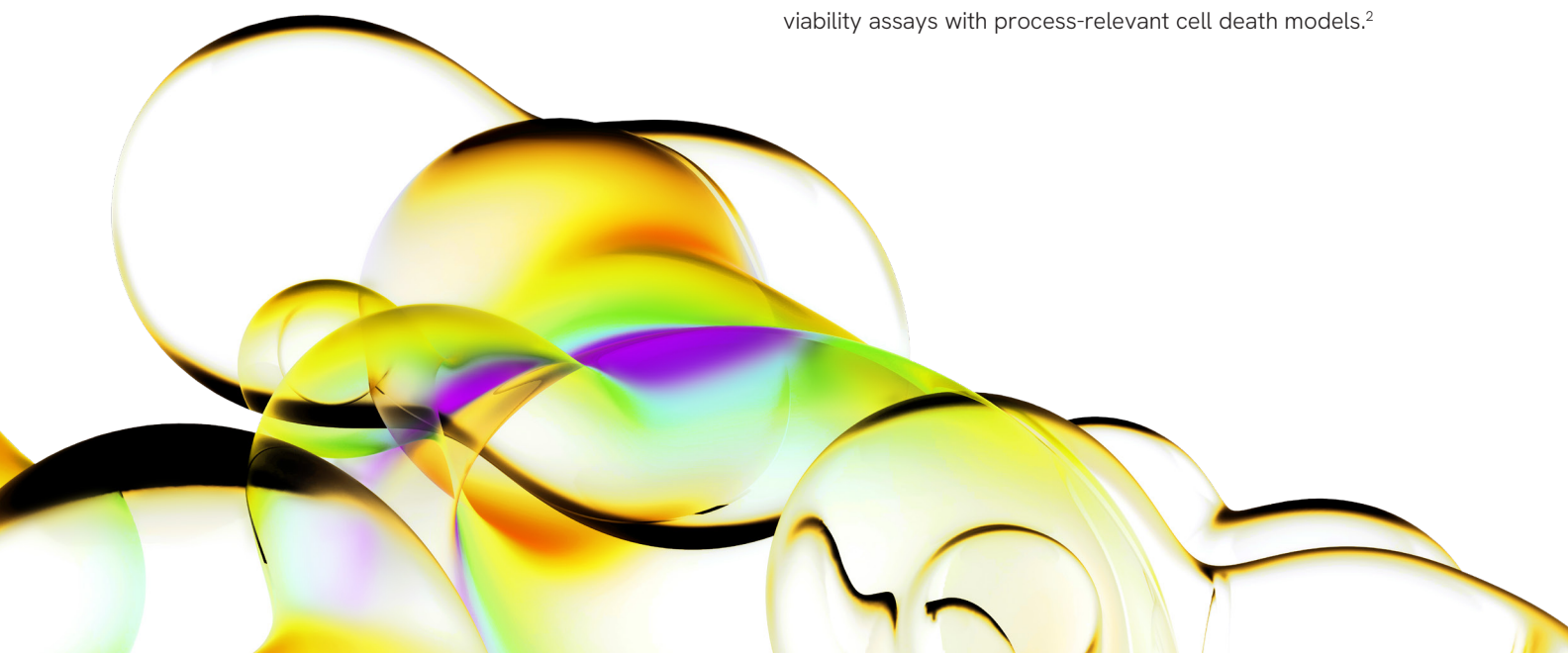
Characterizing AO/PI and AO/DAPI viability assays for cell therapy development using the Cellaca MX high-throughput cell counter.

Introduction

Cell count and viability are two distinct and equally critical quality attributes (CQAs) assessed throughout cell therapy development, manufacturing, and product release testing. Reliable viability measurements are essential for monitoring manufacturing processes, assessing product quality, and supporting release decisions.

Fluorescence-based membrane integrity assays, including acridine orange/propidium iodide (AO/PI) and acridine orange/4',6-diamidino-2-phenylindole (AO/DAPI), are widely used to determine cell viability by identifying membrane-compromised dead cells. AO molecules are cell permeable and can enter the cells and bind with DNA and RNA by intercalation. Both PI and DAPI molecules are membrane-impermeant dyes that can only enter membrane-compromised cells. Although both PI and DAPI fluorescent nucleic acid stains have shown comparable performance using heat-killed (HK) cell death models in a previous publication,¹ differences have been reported when evaluating samples from different stages of cell therapy bioprocesses. This highlights the importance of evaluating viability assays using cellular models that better reflect process-relevant conditions.

Revvity's Cellaca™ MX high-throughput cell counter combines automated brightfield and fluorescence imaging with an advanced cell counting analysis workflow in the Matrix software, allowing for rapid, simultaneous assessment of cell count and viability using fluorescence-based viability assays. In this application note, we present a study using the Cellaca MX cell counter to evaluate the performance of AO/PI and AO/DAPI viability assays with process-relevant cell death models.²



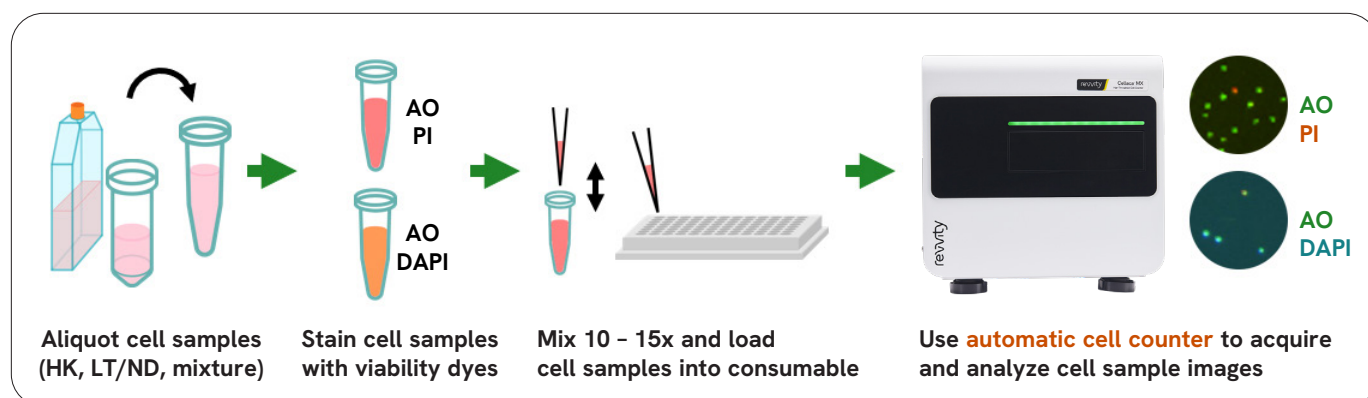


Figure 1: A typical workflow for cell viability measurements. First published in the Journal of Fluorescence, volume 34, pages 2263-2278, 2024 by Springer Nature.²

Experimental design

In this study, two different Jurkat cell death models were tested: heat-killed (HK) cells generated by thermal treatment, and low-temperature/nutrient-deprived (LT/ND) cells generated by storage at 4 °C under nutrient-limited conditions. HK cells represent a traditional low viability quality control material, whereas LT/ND cells more closely mimic the gradual loss of viability observed during cell processing.

Cell samples were stained using AO/PI and AO/DAPI dual-fluorescence viability dyes; Revvity's AO/PI viability dye (part number: [CS2-0106-5mL](#)) was used for AO/PI staining, while both commercial and custom AO/DAPI formulations were evaluated. Following staining, samples were loaded into Revvity's high-throughput cell counting plates* (part number: [CHM24-A100-001](#)) and analyzed on Revvity's Cellaca MX high-throughput cell counter* (part number: [MX-SYS1](#)) to determine total, live, and dead cell counts, from which cell viability was calculated. The overall workflow for cell viability measurements, including sampling, staining, imaging, and data analysis, is shown in Figure 1.

A series of experiments were used to assess the performance of the AO/PI and AO/DAPI viability assays. These experiments evaluated whether each assay measured process-relevant dead-cell populations, produced stable viability measurements over time, and provided comparable viability readouts to the targeted viabilities across the viability detection range.

Results

Observation of cell viability under LT/ND conditions

To evaluate the performance of AO/PI and AO/DAPI viability assays under process-relevant conditions, Jurkat cells with >90% initial viability were stored at 4 °C under nutrient-deprived conditions and measured daily from day 0 to day 4. Total cell concentrations were comparable across both assays; however, viability measurements were comparable on day 0, but differed by 11 – 18% between AO/PI and AO/DAPI after day 1, confirming previously reported anecdotal observations (Figure 2).

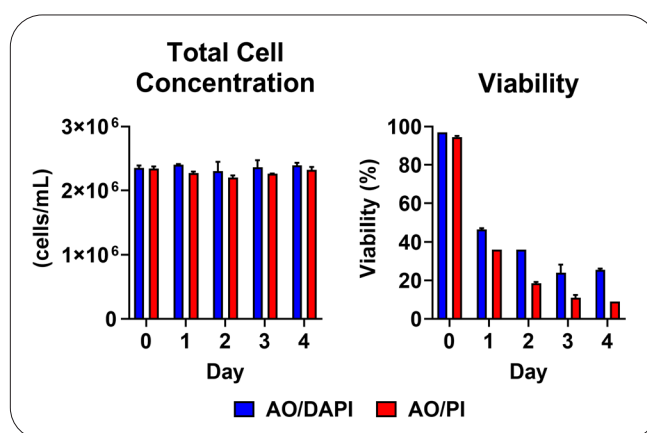


Figure 2: Observations of total cell concentration and cell viability of Jurkat cells under LT/ND conditions, using AO/PI and AO/DAPI viability dyes. First published in the Journal of Fluorescence, volume 34, pages 2263-2278, 2024 by Springer Nature.²

*The Cellaca MX high-throughput cell counter used in this experiment has been superseded by the Cellaca PLX image cytometer (part number: [PLX-SYS1](#)), and the high-throughput cell counting plates used have been superseded by Revvity's Ergonomic high-throughput cell counting plates (part number: [CHM24-A100-098](#)).

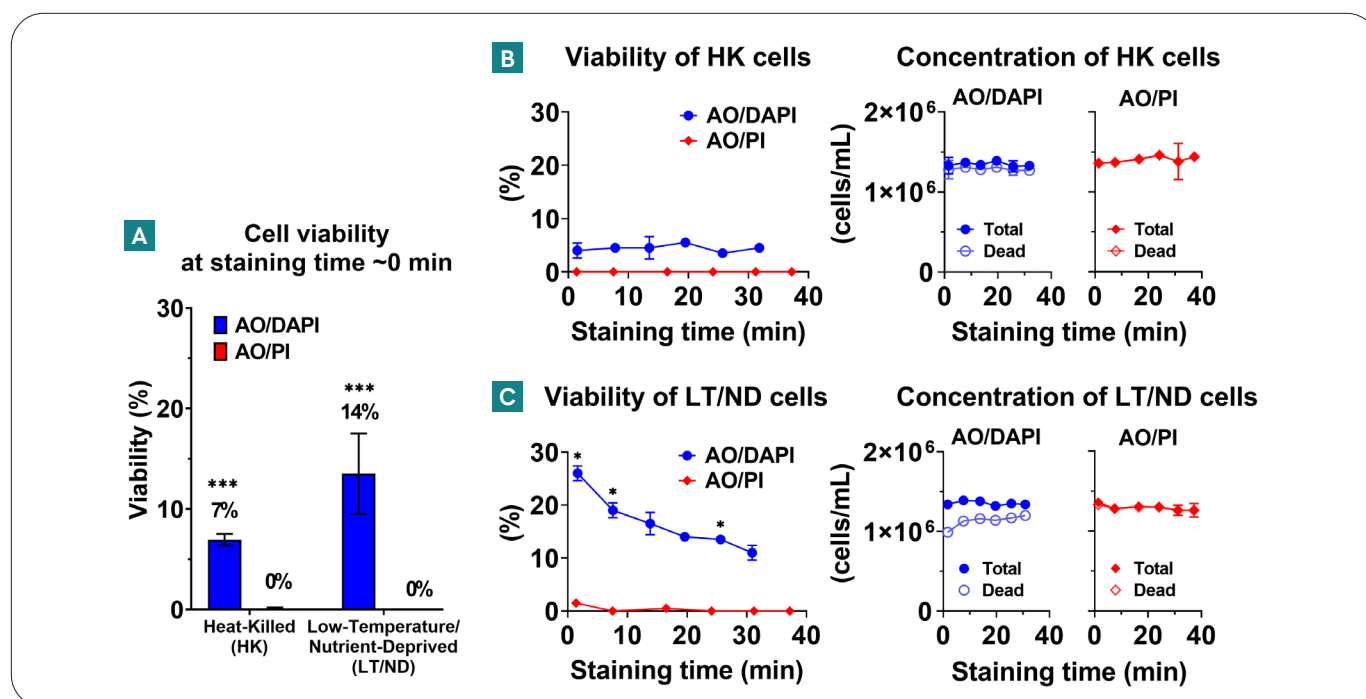


Figure 3: Comparison of cell viability and concentrations between AO/PI and AO/DAPI for HK and LT/ND cell death models. (a) Cell viability at ~0 min of staining time for HK and LT/ND dead Jurkat cells. Total, dead cell concentrations, and viability over the ~40 min staining time for (b) HK and (c) LT/ND cells. First published in the Journal of Fluorescence, volume 34, pages 2263-2278, 2024 by Springer Nature.²

Staining time-dependent effects on viability measurements

The effect of staining time on viability measurements was evaluated using both HK and LT/ND Jurkat cells. LT/ND cells were stored at 4 °C for 21 days to ensure complete loss of viability prior to analysis. Cells were stained with AO/PI or one of four AO/DAPI formulations and analyzed using the Cellaca MX high-throughput cell counter. Baseline measurements were acquired immediately after staining (~0 min; n = 12 replicates). Separate time-course measurements were conducted at ~7 min intervals for up to 40 min (n = 2 per time point).

Total and dead Jurkat cell concentrations along with cell viabilities, with respect to the staining time for both cell death models, are shown in Figure 3. In the baseline measurement, AO/PI consistently measured approximately 0% viability for both HK and LT/ND cell samples, whereas AO/DAPI measured viabilities of ~7% (HK) and ~14% (LT/ND). Over the time course, AO/PI measurements remained stable for both HK and LT/ND cells, with consistent total and dead cell counts and viabilities of ~0%. In contrast, AO/DAPI measurements showed distinct staining profiles in HK and LT/ND cells. In HK samples, AO/DAPI measurement showed consistent total and dead cell concentrations over time with the measured viabilities consistently at ~5%.

In LT/ND samples, AO/DAPI measurements showed a time-dependent increase in dead cells, leading to progressively lower viability results from ~26% to ~11% over ~30 minutes of staining time. These results show that, within the tested staining window, AO/PI measurements remained stable over staining time, whereas AO/DAPI measurements might be time dependent. The follow-up study confirmed that neither HK nor LT/ND cells proliferated over 5 days, proving the higher AO/DAPI viability values reflected staining artifacts, not live cells.²

Viability assessment across the measurement range

To evaluate viability assays across a range of cell viabilities, mixtures of viable and LT/ND Jurkat cells were prepared to generate cell samples with target viabilities of 0, 25, 50, 75, and 100%. Cell mixtures were stained with AO/PI and AO/DAPI and analyzed using the Cellaca MX cell counter immediately after staining (~0 min; n = 4 per mixture). Measured viability was compared with the expected viability of each cell mixture.

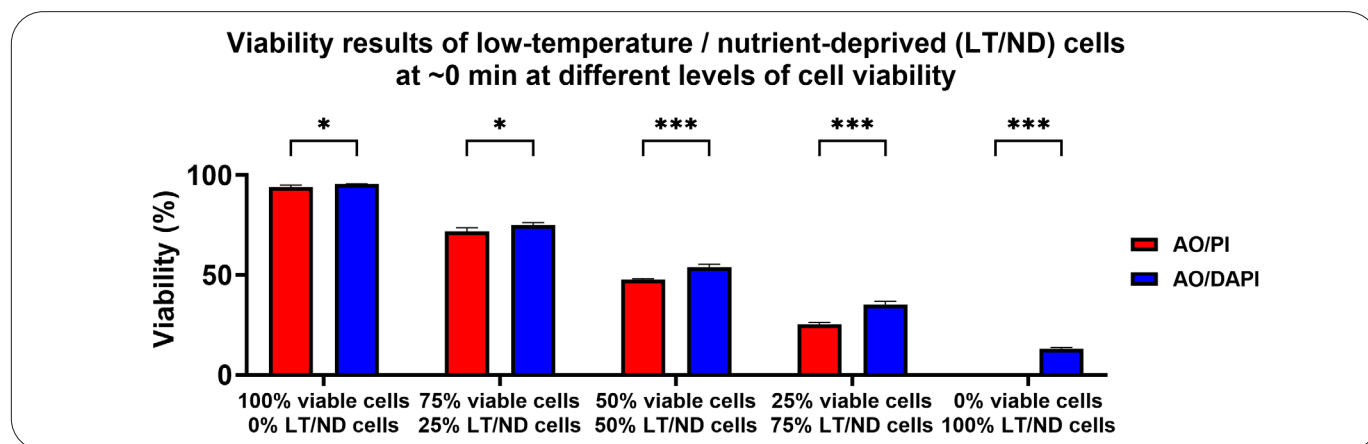


Figure 4: Comparison of AO/PI and AO/DAPI viability measurements at ~0 min of staining time across five cell mixtures with target viabilities of 0, 25, 50, 75, and 100%. First published in the *Journal of Fluorescence*, volume 34, pages 2263-2278, 2024 by Springer Nature.²

At higher viability levels (100% and 75%), measured viabilities were comparable between the two staining methods, with differences of only approximately 2–3% (Figure 4). However, as the proportion of non-viable cells increased, agreement between the assays decreased. At 50%, 25%, and 0% target viability, differences increased to approximately 6–13%, with AO/DAPI consistently measuring higher viability than AO/PI. These viability differences observed at the initial staining timepoint (~0 min) were statistically significant for all cell mixtures.

Conclusion

Cell count and viability measurements are two CQAs assessed throughout cell therapy product development. Selection of an appropriate viability assay is essential for generating reliable data for the development, manufacturing, testing, and release of cell therapy products.

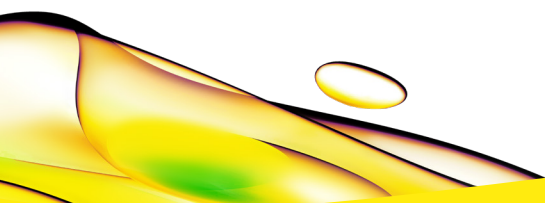
In this study, two fluorescent-based viability assays, AO/PI and AO/DAPI, were evaluated on the Cellaca MX high-throughput cell counter. Across the evaluated conditions, AO/PI provided stable viability measurements that were

largely independent of staining time and closely reflected expected viability values, particularly in low-viability samples. AO/DAPI produced comparable results for healthy cell samples but exhibited time-dependent staining behavior that might result in higher viability readouts from those samples enriched with process-relevant dead cells within a short incubation time.

Together, this study also showcased how the [Cellaca MX high-throughput cell counter](#) could be employed to help characterize and select a viability assay for cell therapy workflows.

References

1. Chan, L.L., Wilkinson, A.R., Paradis, B.D. et al. Rapid Image-based Cytometry for Comparison of Fluorescent Viability Staining Methods. *J Fluoresc* 22, 1301-1311 (2012). <https://doi.org/10.1007/s10895-012-1072-y>
2. Huang, Y., Watkins, R., Patel, S. et al. Practical Characterization Strategies for Comparison, Qualification, and Selection of Cell Viability Detection Methods for Cellular Therapeutic Product Development and Manufacturing. *J Fluoresc* 34, 2263-2278 (2024). <https://doi.org/10.1007/s10895-023-03382-1>



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