Accurate, High-Throughput Cell Viability and Purity Measurements with Aura CL

Introduction

The FDA references USP chapter <1046> to ensure patient safety by providing recommendations for high-quality manufacturing of cell therapies. This chapter lays out guidelines for several release tests, including product purity. Tracking the percentage of viable cells in a product is a key product purity parameter, as are limits on process and particulate impurities within it.[1]

The assessment of cell viability and apoptosis has traditionally relied upon flow cytometry and manual hemocytometry techniques.[2] These techniques face several key challenges in conducting accurate and high-throughput viability assessment of cell therapy products. First, fluidic lines and microfluidic channels often clog when handling the high concentration of cells used in all cell therapy products, disrupting the operation of the instrument and leading to a loss of precious samples. In addition, these methods are user-intensive and relatively low-throughput, creating the potential for analytical bottlenecks that slow progress. Furthermore, the distinction between cellular and non-cellular material using standard viability techniques in both flow cytometry and hemocytometry is incredibly complicated, which can mislead researchers into confounding cell viability results with product purity issues. Finally, imaging-based cytometers can also miss protein aggregates or other particles with low refractive index and will not be able to be detected from the liquid background.[3]

Thus, providing rapid, reliable, and high-throughput viability and purity analysis for cell therapies has proven challenging – until now.

Aura CL™, powered by Backgrounded Membrane Imaging (BMI) and Fluorescent Membrane Microscopy (FMM), enables high-throughput, low volume, accurate cell viability assays for the assessment of your cell therapy product. Aura CL, combined with the advanced analytical capabilities of Particle Vue software, accurately, rapidly, and reliably assess cell viability in a 96-well format. So, whether you are measuring viability of your iPSC seed population or your expanded population of T cells, you can now perform high-throughput, rapid, and accurate viability assessment while simultaneously understanding the purity state of your product.

This application note introduces a simple, two-step cell labeling method that identifies non-viable (dead) from viable (live) cells with a high degree of specificity and accuracy using Aura CL. Results were validated with a thorough comparison to the most common cell viability techniques. In addition, we extend this technique beyond characterizing live and dead cells to facilitate simultaneous identification of other particles and impurities present in cell therapy products including proteinaceous and non-cellular subvisible particles. Last, we demonstrate the universality of using Aura CL to perform other cell assays including apoptosis.

Methods

Preparation of Cells

Cultured Jurkat T cells (ATCC, catalog no. TIB-152) and THP-1 monocytes (ATCC, catalog no.TIB-202) were maintained under standard culture conditions in appropriate culture media supplemented with 10% FBS, L-Glutamine, penicillin, and streptomycin. Cells were harvested at approximately 85-90% confluency and assessed for viability. Non-viable cell populations were prepared by exposing T cells to multiple freeze-thaw cycles (>5 cycles). Prior to analysis, all cell populations were washed in cold PBS and adjusted to a concentration of $1x10^6$ cells/mL.

Assessment of Cell Viability using DAPI

DAPI (4′,6-diamidino-2-phenylindole) is a DNA-specific stain that exhibits an increase in fluorescence upon binding. It is cell impermeant at low concentrations (<0.05 µg/mL) but can label live cells at higher concentrations (>1.0 µg/mL)[4]. However, an increase in plasma membrane permeability is a hallmark of non-viable cells, allowing DAPI staining even at low concentrations. This differential staining property was used to specifically distinguish non-viable cells from the total population using FMM. This property provides the basis of many viability assays such as propidium iodide for flow cytometry or trypan blue for manual hemocytometry.[2,5]

Aura CL cell viability assay utilizes a two-step staining process (Figure 1). First, 1 x 10^6 cells/mL were stained in solution using a final concentration of 0.05 µg/mL DAPI to determine the number of non-viable cells in the sample. The cells were stained for 10 minutes in the dark before 50 µL/well were applied to a backgrounded black membrane plate, vacuumed, dried, and imaged in brightfield, SIMI, and FL2 channels (ex. 376/30 and em. 440/40 nm). Next, the cells were stained on membrane with high concentrations of DAPI, vacuumed, dried, and imaged in brightfield, SIMI, and FL2 to determine the entire cell population. A high concentration of DAPI (2.0 µg/mL) was applied to the sample wells and incubated in the dark for 10 minutes. The plate background was cloned in Particle Vue software in order to analyze the same wells containing previously filtered cell therapy samples. The Particle Vue expression engine was used to identify DAPI positive based on their morphology (minimum Feret of 5–20 µm) and fluorescent properties (average FL2 Intensity of greater than 30), signifying a strong fluorescence signal. Finally, we stained the same population with the fluorescent protein stain, Thioflavin T, at 5 µM (final concentration) for 1 minute and re-imaged the plate using the FL1 channel (ex. 440/40nm and em. 500/40nm).

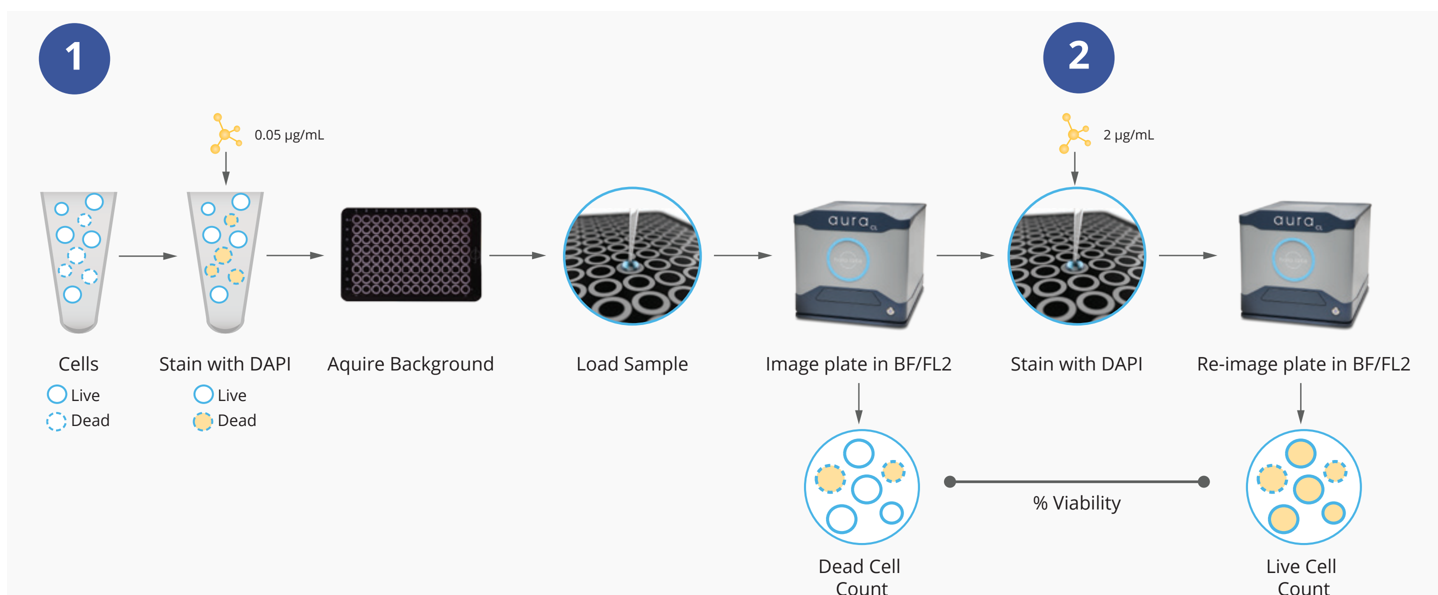


Figure 1: Aura CL cell viability assay overview. Step 1 uses in-solution staining step to identify populations of non-viable (dead) cells using DAPI at a final concentration of 0.05 µg/mL. Step 2 stains the whole cellular population using a high concentration of DAPI (2 µg/mL). DAPI positive cells are identified by their staining intensity in the FL2 channel (FL2 Intensity >30).

To test the validity of this approach, we applied this staining protocol to freshly cultured T cells, THP-1 monocytes, and non-viable T cells, and compared counts to the standard manual hemocytometry trypan blue (TB) exclusion assay, and flow cytometry using propidium iodide (PI).

Assessment of Cell Viability using PI for Flow Cytometry and TB in Hemocytometry

Cultured Jurkat and THP-1 cells were stained using the Dead Cell Apoptosis kit (ThermoFisher, catalog no. V13241) and analysed using the BD Accuri C6 Sampler flow cytometer. Briefly, $1x10^6$ cells were washed in cold PBS, resuspended with 100 µL binding buffer and incubated in the dark with propidium iodide (1 µL). Positive events were identified using the FL3 channel following population gating using forward (FSC) and side scatter (SSC). For manual viability assessment, 50 µL of cultured cells were mixed with 50 µL of 0.4% trypan blue solution (ThermoFisher, catalog no. 1525006), and immediately assessed for non-viable cells using a hemocytometer and a standard light microscope.[1,6]

Assessment of Particle Impurities in Cell Populations.

To determine the proportion and derivation of cell impurities, a mixed population of viable and non-viable T cells were applied to a backgrounded black membrane plate, filtered, and imaged. Following BMI, cells were stained on the membrane with 50 µL of DAPI (2 µg/mL), incubated for 10 minutes in the dark, filtered, and re-imaged using the FL2 channel on Aura CL. Cells were subsequently stained with 50 µL of 5 mM Thioflavin T (ThT), incubated for 5 minutes in the dark, filtered, and finally imaged using the FL1 channel. Data was then analyzed in Particle Vue software v4.0 to assess the population of cells (defined as DAPI+, ThT+), protein and cell debris (defined as DAPI-, ThT+), and non-cellular impurities (DAPI-, ThT-) using an FL1 and FL2 intensity threshold of 30.

Assessment of Cell Status using Annexin V

Aura CL can also rapidly assess the percentage of apoptotic cells using Annexin V conjugated to the fluorescent dye Alexa Fluor 350 (ThermoFisher). Annexin V binds to phosphatidyl serine, a negatively charged phospholipid that flips from the cytosolic side of the plasma membrane to the cellular surface when the cell is apoptotic or necrotic.[7] To investigate the linearity of Annexin V staining, we prepared pre-defined mixes of viable and non-viable T cells by mixing freshly cultured T cells with non-viable T cells, at 0%, 25%, 50%, 75%, and 100% proportions of non-viable cells. Both cell populations were counted before mixing and normalized to a final concentration of $1x10^6$ cells/mL. The resultant cell populations were stained according to the manufacturer's protocol. Briefly, cells were washed in cold PBS and resuspended in 1x Annexin V binding buffer. Annexin V (5 µL) was added to 100 µL of cells, incubated in the dark for 15 minutes, and further diluted in 400 µL of Binding Buffer. Finally, 40 µL of stained cells were added in triplicate, to each well on a backgrounded black membrane plate, subsequently filtered and imaged at an exposure of 7.5 seconds. Annexin V positive cells were identified as cells with an FL2 intensity greater than 3 standard deviations (SD) above the background.

Results

Assessment of Cell Viability using DAPI

As previously mentioned, Aura CL viability assay exploits the differential staining properties of live and dead cells with the nuclear stain DAPI. Using this simple two-step staining method, we can reliably differentiate non-viable cells from the total cell population using the standard calculation:

$$\textbf{\%}\ \textit{Viability} = 1.0 - \left(\frac{\textit{Non Viable Cell Count}}{\textit{Total Cell Count}} \right) \times 100$$

To confirm the validity of this approach, we carried out a comparative analysis of the same cell population using two standard viability assays: manual counting using TB and flow cytometry using PI (Figure 2). Our analysis proves that Aura CL compared favorably, and within error, with these two industry-standard techniques in both cultured T cells and THP-1 monocytes (Table 1).

T cell viability can be determined by the differential uptake of DAPI at two concentrations, 0.05 µg/mL and 2.0 µg/mL. Figure 3 highlights the differential staining properties of viable and non-viable cells within a mixed T cell population.

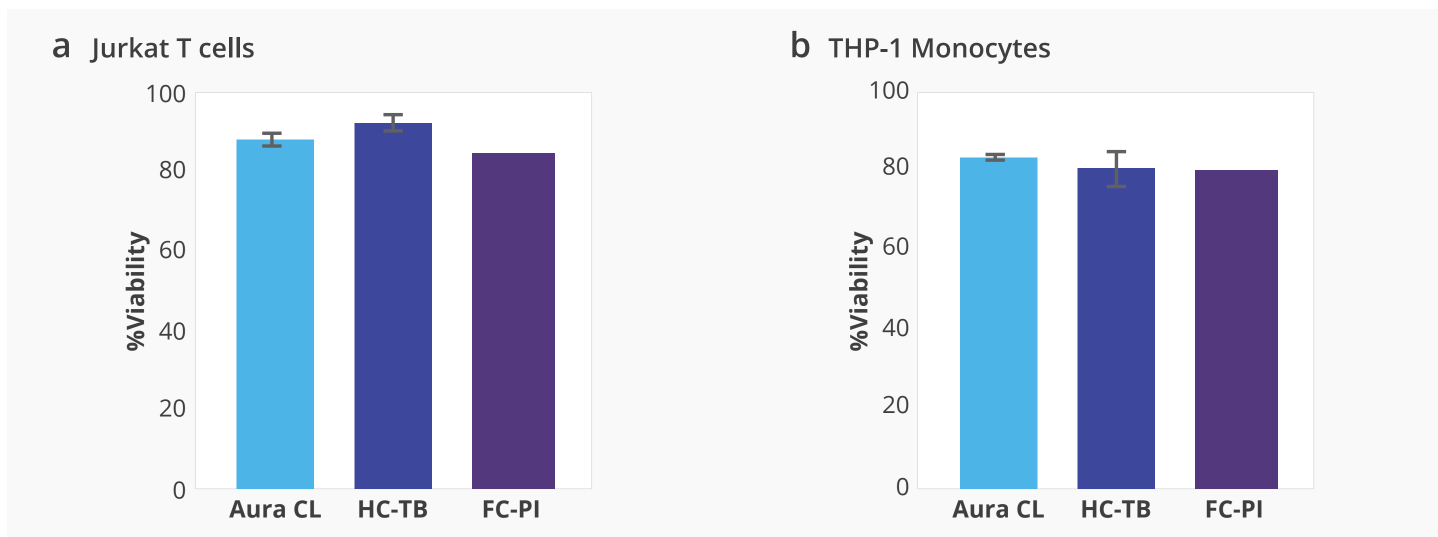


Figure 2: Comparative analysis of the Aura CL viability assay with two industry-standard techniques. The Aura CL viability assay relies on fluorescent membrane microscopy (FMM) and the nuclear stain DAPI (FMM-DAPI). Data generated on Aura CL (n=3) was comparable with both hemocytometry using trypan blue (HC-TB, n=2) and flow cytometry using propidium iodide (FC-PI, n=1) to stain non-viable cells. In each assay, two different cultured cell populations (a) Jurkat T cells and (b) THP-1 monocytes were tested for baseline viability.

Technique	Jurkat	THP-1
Aura CL Viability (n=3)	88.0	83.5
Hemocytometry - Trypan Blue (n=2)	92.3	80.7
Flow Cytometry - Propidium Iodide (n=1)	84.8	79.6

Table 1: Summary table comparing the Aura CL viability assay with a trypan blue exclusion assay and flow cytometry.

We noted a significant size reduction in non-viable cells (7.32 µm, SD±4.36) in comparison to the viable cell population (13.84 µm, SD±7.46). However, we caution against using cell size alone as an indication of cell status and viability.

Determining populations of DAPI positive cells in both groups (non-viable and total cell) allows for the rapid assessment of cell viability. We show two histograms of the same cell population initially stained in solution with DAPI at low concentration (0.05 µg/mL) and further stained on the membrane using DAPI at high concentration (2 µg/mL) (Figure 4). The percentage cell viability on the same filtered population can be determined by comparing the non-viable cell count against the total cell count. Applying an FL-2 intensity threshold based upon DAPI positive events in the total cell population (Figure 4b) and subsequently to the non-viable population (Figure 4a) allows for the accurate assessment of viability (Table 1).

Of particular note is that Aura CL can also determine the proportion of non-cellular debris, such as protein aggregates, within a given cell therapy (Figure 3, unstained particles).

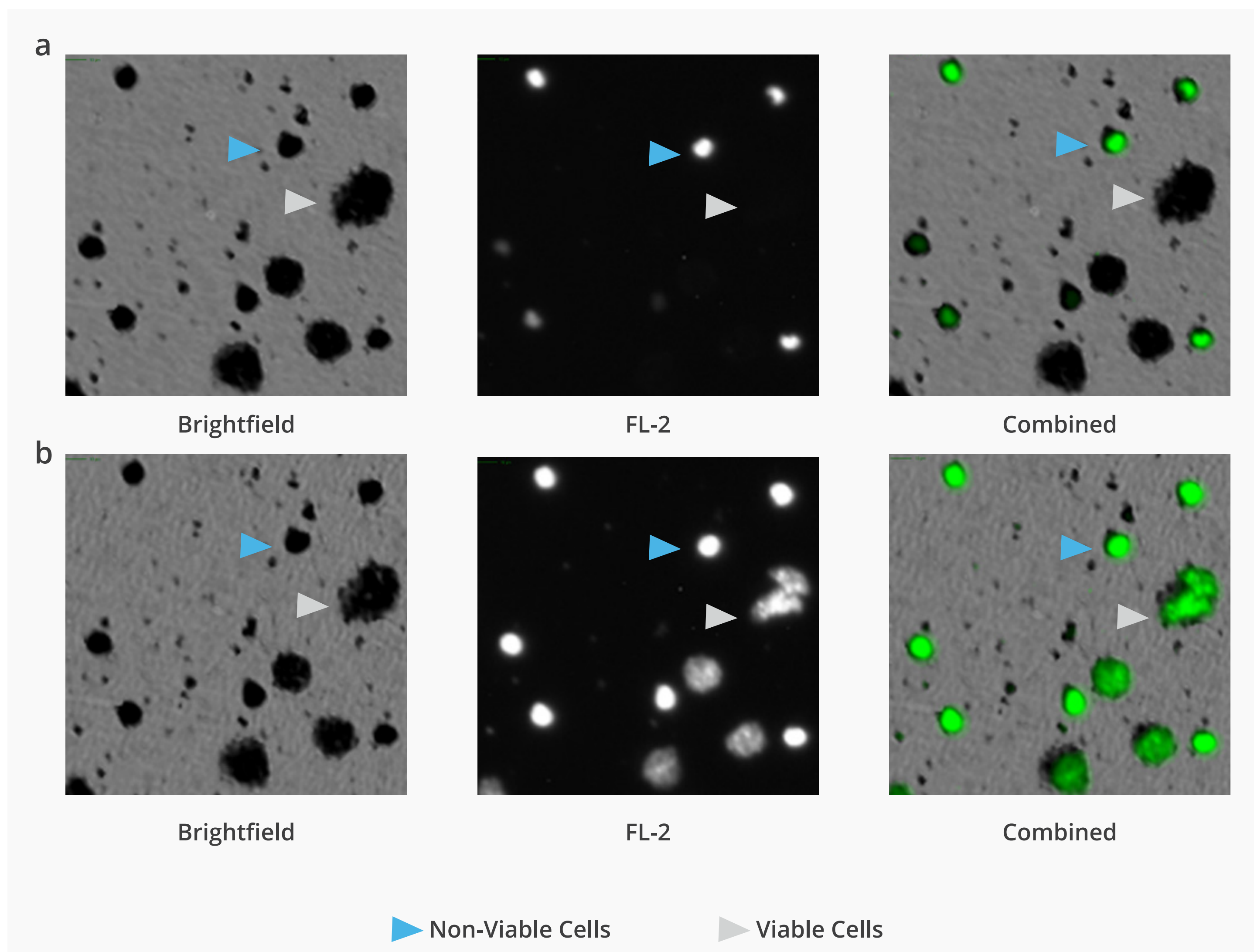


Figure 3: Non-viable cells exhibit greater fluorescent intensity when stained with DAPI at different concentrations. DAPI staining can be used to distinguish between dead (non-viable, blue arrow) and live (viable, grey arrow) cells; (a) at low concentrations (0.05 µg/mL) non-viable cells are preferentially stained with DAPI compared to (b) high concentrations (2.0 µg/mL) of DAPI that stains all cells. Unstained particles like protein aggregates are also clearly evident within the imaged population.

Identification of Non-Cellular Particles and Aggregates in Cell Therapy Products

A key regulatory requirement for any injectable therapeutic is the accurate determination of subvisible particles (SVP) and aggregates that can form during the manufacturing process, providing essential information on product quality and safety.[8] The inherent capability of Aura CL to differentiate between cellular and non-cellular particles is an important development in this area.

The incorporation of FMM in Aura CL enables you to determine the origin of non-cellular particles through the application of Thioflavin T (ThT), a fluorescent dye that binds specifically to protein aggregates and cells (Figure 5). Aura CL can distinguish between viable, non-viable cells, protein aggregate, and non-cellular particulates.

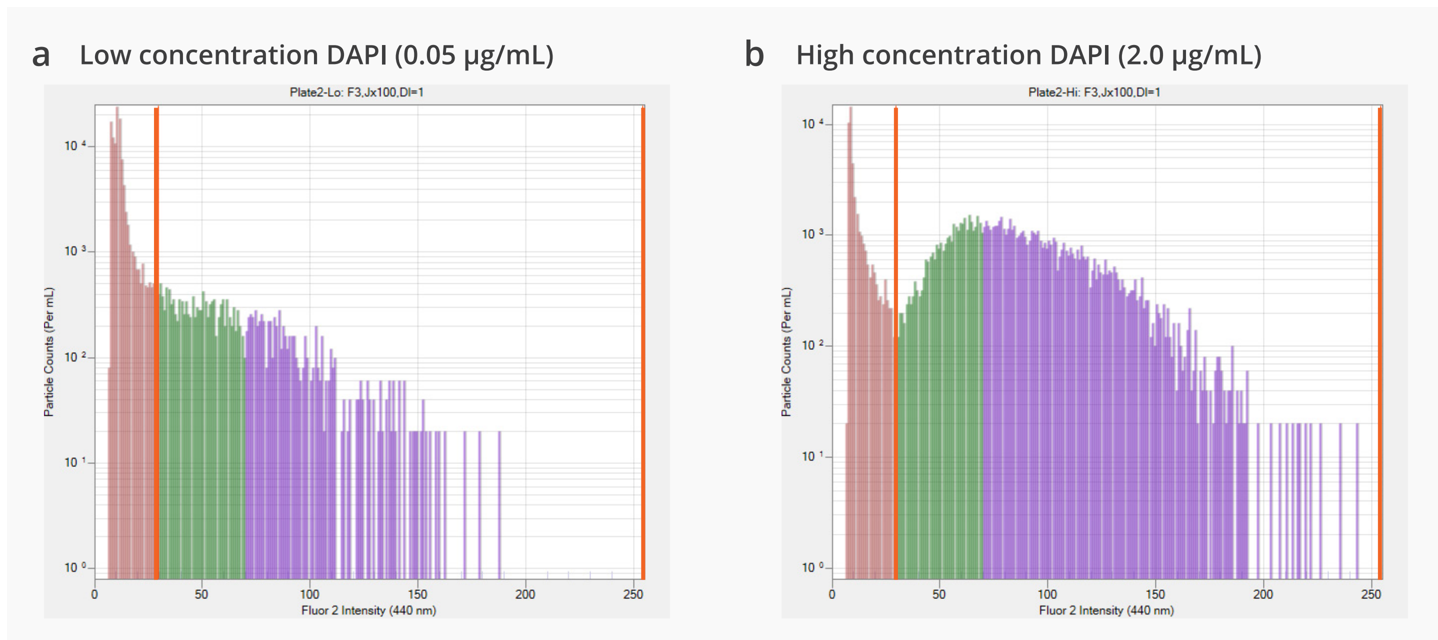


Figure 4: FL-2 (DAPI) intensity histogram. The histogram function in the Particle Vue software enables the identification of DAPI positive populations either stained using (a) a low concentration (0.05 µg/mL) or (b) a high concentration DAPI (2.0 µg/mL). The threshold intensity for positive staining is defined by the whole population staining and applied to the non-viable population allowing for viability assessment.

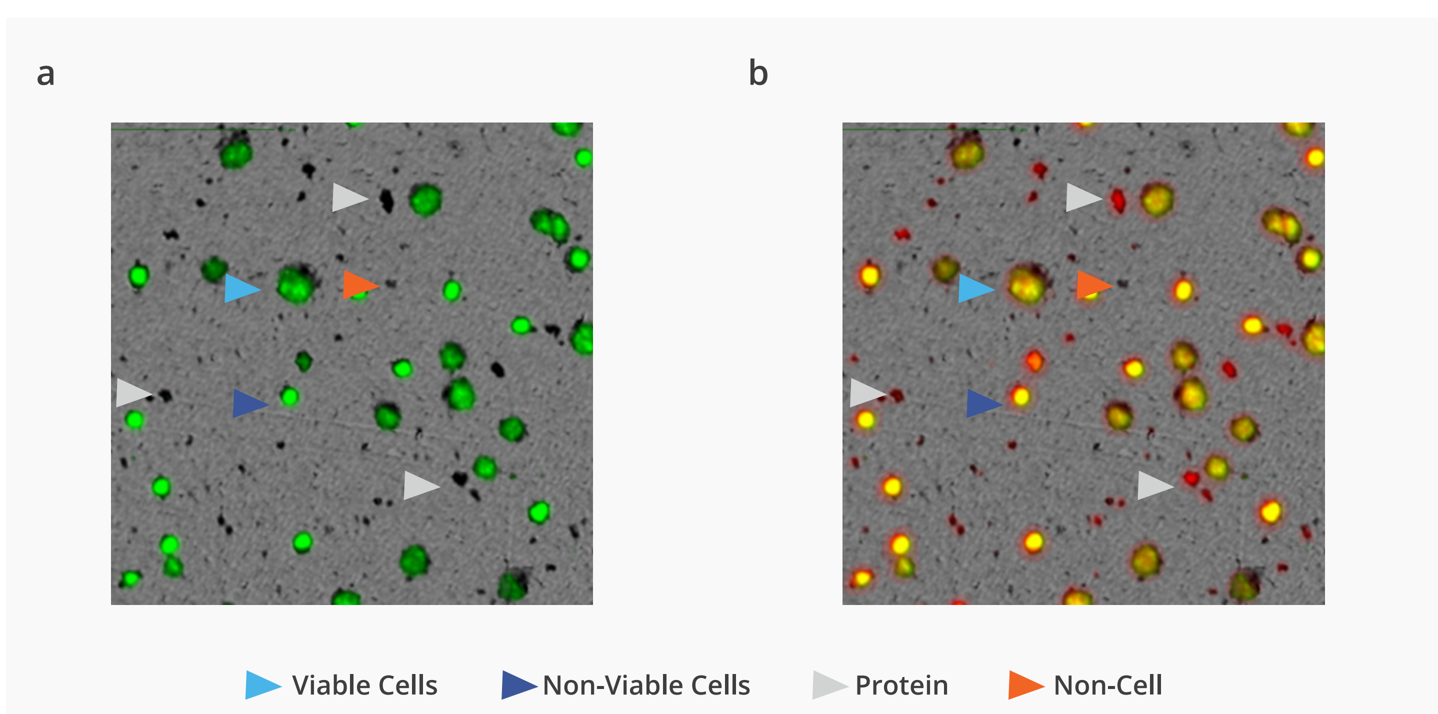


Figure 5: Cell and protein analysis on Aura CL. (a) DAPI is effective at staining nuclei on the membrane enabling the positive identification of cells, viable (orange arrow) and non-viable (purple arrow), allowing for their differentiation from other particles resident on the filtered membrane. (b) Staining with ThT allows for additional evaluation through staining both cells and protein aggregates, thus enabling the distinction between cells, protein aggregates, and other non-cellular material.

Furthermore, one can examine the contribution of each particle type to the total number of particles within a given cell therapy product (Figure 6). Cellular particles exhibit strong dual fluorescence in both the FL1 channel (ThT) and FL2 channel (DAPI), whilst protein particles will stain positively for ThT only. Additionally, non-cellular/non-proteinaceous material can also be defined by their relative negative ThT and DAPI. In this analysis, we identified all particles with an equivalent circular diameter (ECD) greater than 5 µm in a mixed population of viable and non-viable T cells and plotted the FL1 intensity (ThT) against FL2 intensity (DAPI). Applying a quadrant threshold identified specific sub-populations based on their relative staining properties. In this example, T cells accounted for 78.2% of the total population, whilst protein and non-cellular material accounted for 17.2% and 4.5% respectively (Figure 6). This analysis enables easy identification and interpretation of mixed particle populations within a given cell therapy product.

The expression engine within Particle Vue software allows for rapid analysis of particle sub-populations using all analysis parameters, including particle size and fluorescence intensity. Using the expression engine, we were able to determine the percentage of all the particle subpopulations within the freshly cultured T cells (untreated) and non-viable T cells treated with several rounds of freeze-thaw cycles (Figure 7). There was an observed difference in percentage viability, as a function of the total cell population, of untreated (81.5%) and treated (10.5%) T cells. Interestingly, cells that have been treated with multiple rounds of freeze-thaw cycles exhibit an increase in protein particles when compared to untreated (cultured) cells. This could be explained by the propensity for albumin, and other serum proteins to aggregate and cells to lyse in response to multiple freeze-thaw cycles).[9]

The relative contribution of particle subpopulations can be identified with Aura CL using a combination of brightfield, SIMI, and 2-channel fluorescence. This provides the ability to define particles in a complex mixture like a cell therapy product. Particles that exhibit high SIMI intensity and no fluorescence (Figure 7i) can be described as non-biological in nature (e.g. plastic) that is derived from the environment or manufacturing process. Protein particles and cells are both negative for SIMI and positive for fluorescence either with ThT alone (protein, Figure 7ii) or both ThT and DAPI (cells; Figure 7iii and 7iv).

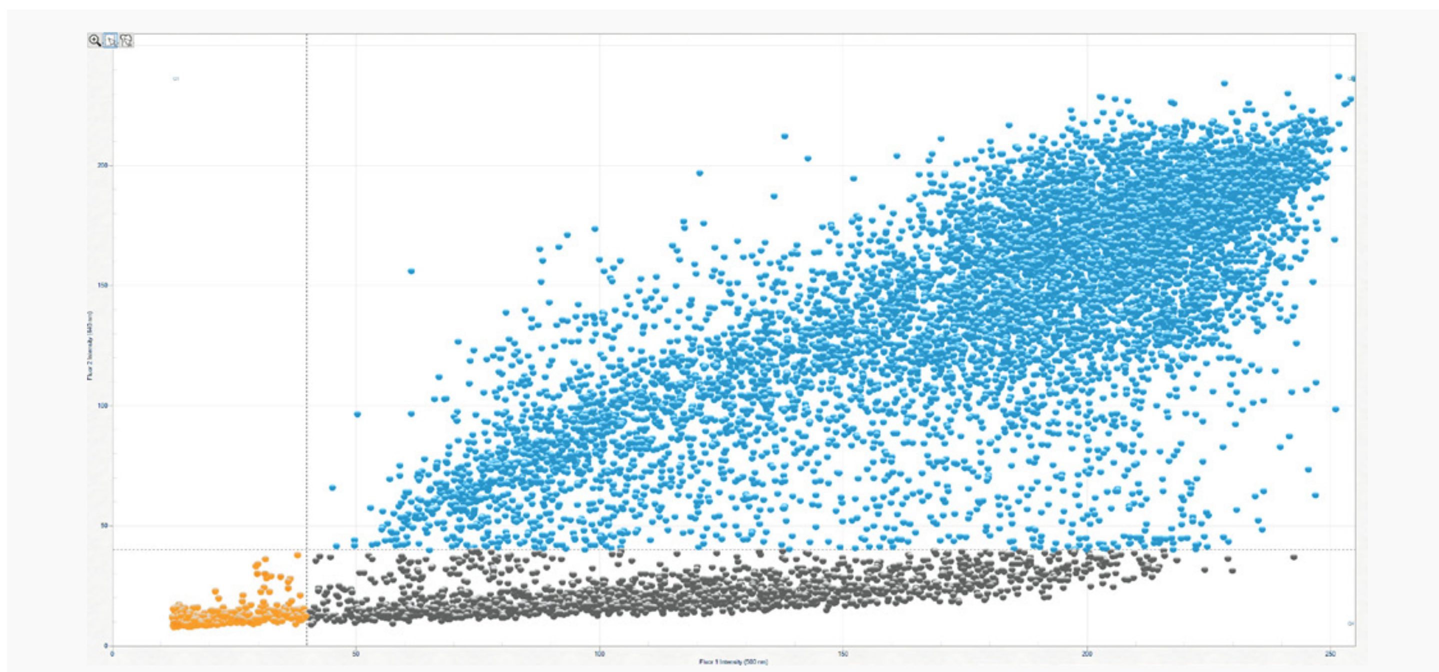


Figure 6: 2D scatterplot of FL1 (Thioflavin T) versus FL2 (DAPI). With the dual staining approach, cells (blue) exhibit positivity for both Thioflavin T (protein) and DAPI (DNA), whilst protein aggregates (black) are positive for only Thioflavin T. Dual negative, non-cell/protein particles (yellow) have little or no significant staining with either stain. Data shown accounts for all particles greater than 5 µm ECD.

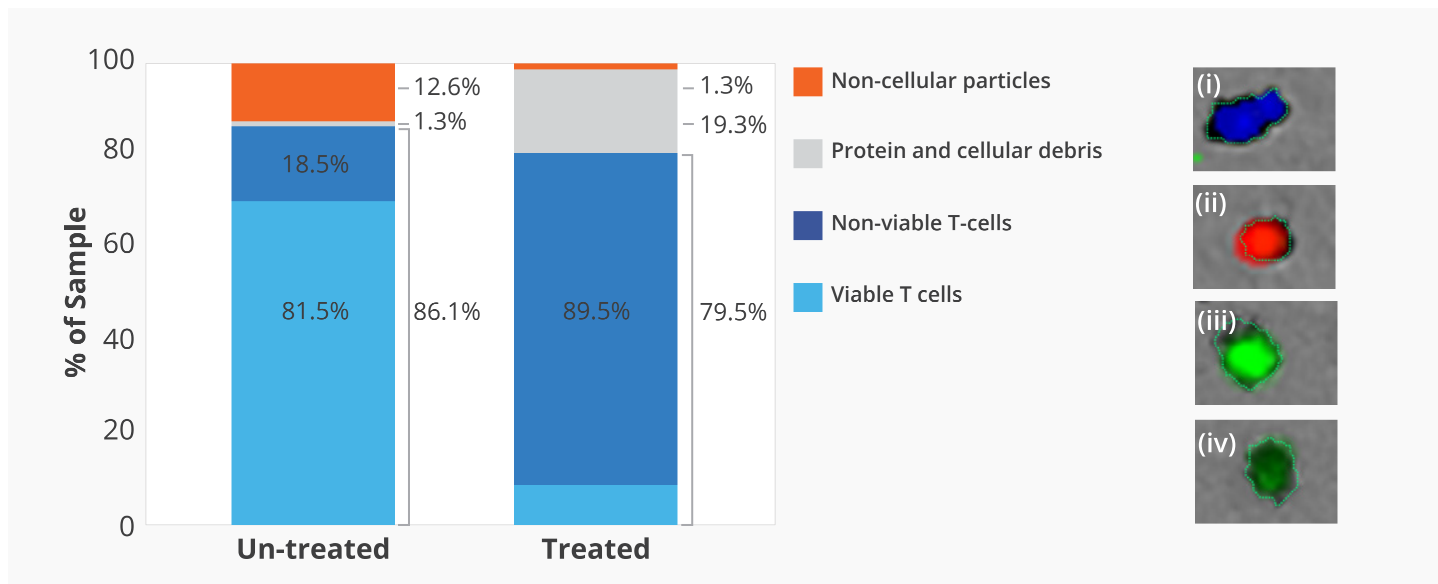


Figure 7: The expression engine tool can be applied to count an entire dataset. The relative contribution of particle subpopulations can be identified using the expression engine tool available with Particle Vue software. Untreated T cells exhibit a different population profile compared to T cells treated with repeated freeze-thaw cycles. Representative fluorescent images of individual particles are shown where (i) non-cellular particles exhibit a high SIMI intensity (blue), (ii) protein particles are positive for ThT (red), and (iii) non-viable (bright green) and (iv) viable cells (green) are positive with DAPI.

Assessment of Cell Status using Annexin V

Aura CL can be adapted to the most common cellular assays. Here, we adapt a commonly used single-cell apoptosis assay that uses Annexin V for flow cytometry to Aura CL. We stained mixed populations of viable and non-viable T cells with Annexin V and analyzed both samples with BMI and FMM. Annexin V positive cells were detected in the FL2 channel (Figure 8a). The advanced analytical tools in Particle Vue v4.0 distinguished apoptotic from non-apoptotic cells with high a degree of accuracy. Apoptotic cells defined as cells with a FL2 intensity greater than 3 SD above the background and normalized to the 100% non-viable cell population. The assay results were linear when we compare different mixed populations of viable and non-viable T cells, with an R^2 of 0.9974 (Figure 8b), highlighting the increased utility of Aura CL.

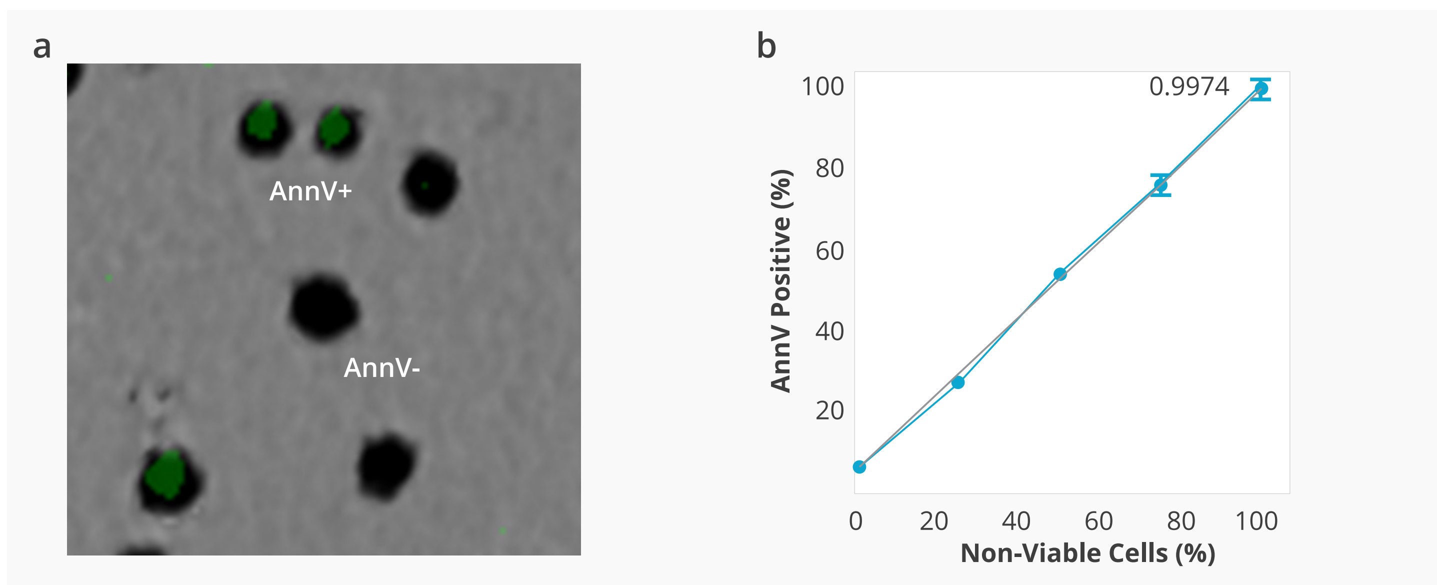
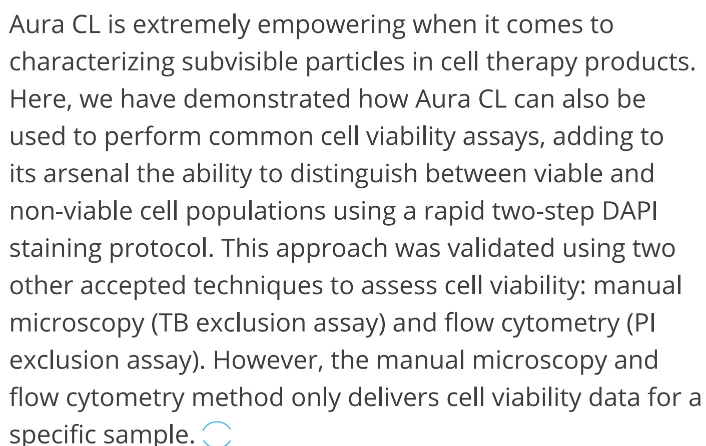


Figure 8: Apoptotic and non-apoptotic cells can be differentiated using Annexin V staining on Aura CL. (a) A representative combined image of a mixed population of Annexin V negative (AnnV-, non-apoptotic) and Annexin V positive (AnnV+, apoptotic) T cells. (b) The linear relationship of mixed populations of apoptotic/non-apoptotic T cells against percentage Annexin V positive cells (n=3).



Conclusion

Aura CL is extremely empowering when it comes to characterizing subvisible particles in cell therapy products. Here, we have demonstrated how Aura CL can also be used to perform common cell viability assays, adding to its arsenal the ability to distinguish between viable and non-viable cell populations using a rapid two-step DAPI staining protocol. This approach was validated using two other accepted techniques to assess cell viability: manual microscopy (TB exclusion assay) and flow cytometry (PI exclusion assay). However, the manual microscopy and flow cytometry method only delivers cell viability data for a specific sample.

AURA CL:

- Delivers protein and non-protein information in the same sample
- Provides accurate counts for viable and non-viable cells, total cells, non-cellular protein aggregates, and non-cellular particles in a single assay
- Detects apoptotic and necrotic cells using Annexin V staining providing a high-throughput, low volume, fluidics–free assay
- Characterizes the viability and purity of any cell therapy product within a few hours in a 96-well format with only 40 µL of sample

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