Evaluate Cell Therapy Product Purity with Aura CL

Introduction

With the advent of clinical-grade CAR-T cell therapies and the recent FDA approval of two autologous CAR-T cell therapies, Yescarta® and Kymriah®, the ability to differentiate contaminating particles from the manufacturing process is a key challenge.[1] Differentiating cells from other types of subvisible particles in the cell therapeutic is virtually impossible given the inherent challenges in characterizing cells using traditional particle analyzers and flow imaging techniques, combined with the relatively low throughput of flow cytometry. A recent study examining the use of the pharmacopeial methods, light obscuration (LO), and flow imaging (FI) for cell analysis found that the cell counting accuracy was only 60% and 50%, respectively.[2] It was postulated that out-of-focus cells in the tall 300 µm fluidic channels for these systems and cell clumping accounted for the massive drop in counting, sizing, and morphological accuracy.

Quantifying subvisible particle content in cellular therapies is incredibly difficult since cells themselves are subvisible in nature.[3] The inherent complexity in the manufacture and supply of CAR-T cell therapies, from collection, viral transduction, expansion and storage, results in greater potential for the formation of large cell and non-cell aggregates affecting clinical safety and efficacy outcomes.[4] As a part of the manufacturing process, a common practice is to use Dynabeads™ for the purposes of enrichment and isolation of CAR-T cells.[5] The beads must be removed from the final product to ensure product purity to avoid being considered a non-biological contaminant along with fibers, plastics, and rubber. With the importance of clinical safety and efficacy outcomes, the key challenge remains being able to count, size and differentiate these sub-visible particles (e.g., Dynabeads) from the therapeutic cells, themselves a sub-visible particle.

Aura CL™ enables cell therapy product purity assessment by combining Backgrounded Membrane Imaging (BMI) with Fluorescence Membrane Microscopy (FMM). This novel particle identification method identifies, categorizes, and further scrutinizes the most common particles in the cell therapy product. It uses established extrinsic fluorescent dye chemistries to distinguish between cellular (e.g., CAR-T cells), non-cellular (e.g., proteins and lipids), and non-biological contaminants (e.g., residual Dynabeads and fibers) and quantify their presence.

This application note illustrates the dynamic capabilities of Aura CL to identify, count, and characterize subvisible particle contaminants introduced in the cell therapy manufacturing process from CAR-T cells. Here, we demonstrate the system's compatibility with well-established fluorescent labeling methods for easy identification of cell and subvisible particles in assessing product purity.

Method

CAR-T cells were mixed with Human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, catalog no: 11131D) and 50 µL of the resultant suspension loaded onto a backgrounded membrane plate that was then imaged using Brightfield and SIMI. As previously described in Application Note 12, the sample was subsequently stained with DAPI (2 µg/mL) or Hoechst 33342 (10 µg/mL) to specifically stain for cellular DNA and imaged using the FL2 channel. In addition, samples could also be counterstained with Thioflavin T, which specifically labels protein aggregates, and detected using FMM in the FL1 channel.

Results

Imaging and Analyzing CAR-T Samples Using BMI and FMM

Analysis of the CAR-T therapy on Aura CL begins with the BMI well image, where high contrast, high resolution images provide the landscape to count and size the particles in your cell therapy product. The incorporation of two fluorescence channels provides the opportunity for dual-color experiments, combined with BMI and SIMI, to enable the complete characterization and identification of different subvisible particles. Figure 1 highlights different types of subvisible particle that can be identified using Aura CL. A variety of subvisible particles were imaged, including CAR-T cells (Figure 1a), residual Dynabeads in the sample (Figure 1b), and lysozyme protein aggregates (Figure 1c),

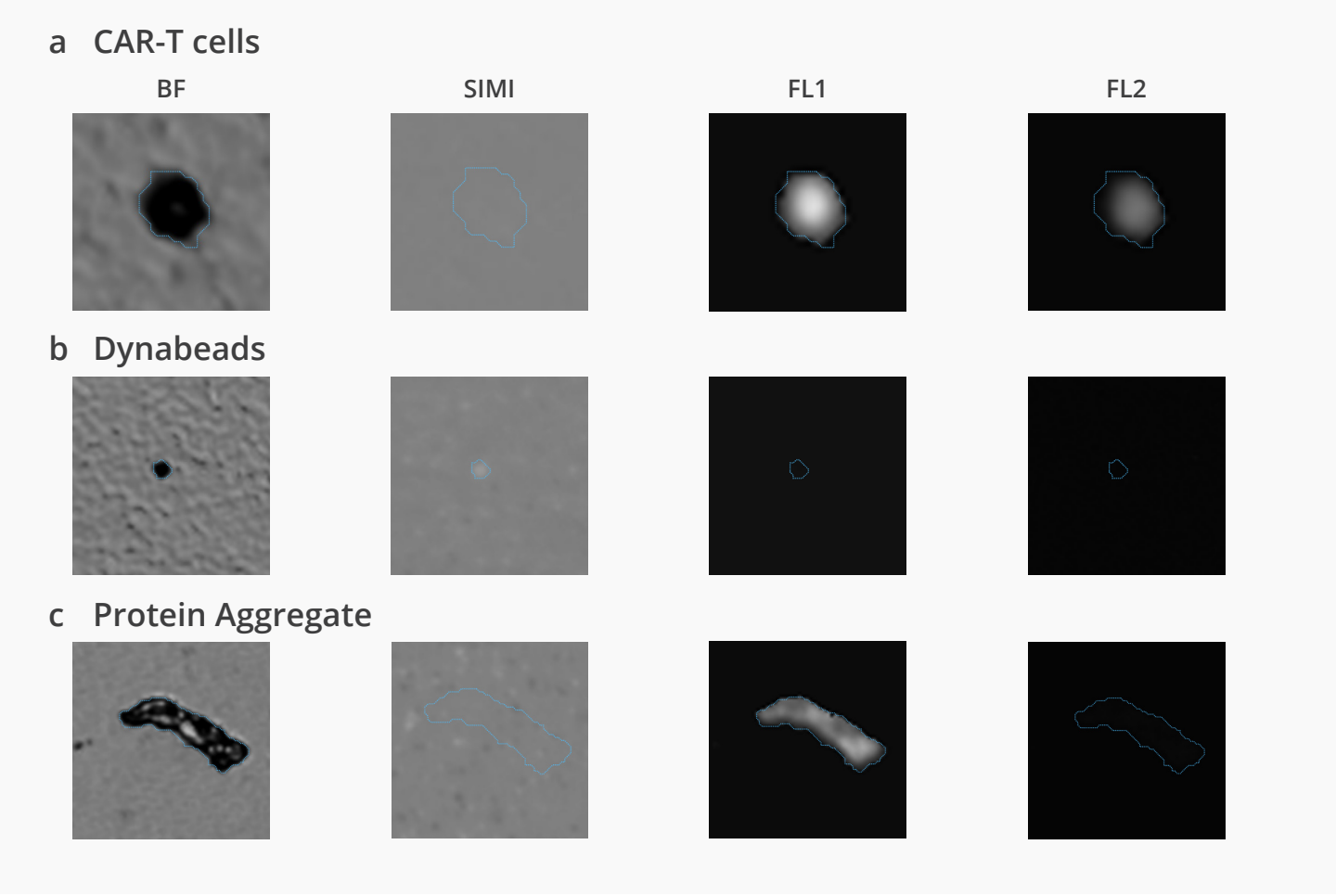

Figure 1: Subvisible particles as imaged on Aura CL. All sub-visible particles (a) CAR-T cells, (b) Dynabeads and (c) lysozyme protein aggregates were stained for protein and DNA and imaged on a black membrane in Brightfield, SIMI, and FL1 (Protein) and FL2 (DNA) channels.

using brightfield (BF), SIMI, FL1, and FL2 following the application of a protein and DNA stain, respectively.

Similar to the Dynabeads, CAR-T cells demonstrate good contrast in brightfield using BMI. In addition, CAR-T cells have low side illumination and are positive for both protein (FL1) and DNA (FL2). Conversely, Dynabeads demonstrated higher SIMI intensity and low protein (FL1) and DNA (FL2) staining intensity in contrast to protein aggregates which were polymorphic exhibiting variable brightfield intensity, low SIMI, positive for the protein stain (FL1), and negative for the DNA stain (FL2). Thus, differentiating between Dynabeads, cells, and protein aggregates in a complex sample using Aura CL is fast and specific due to the built-in orthogonality of using morphology characteristics from brightfield imaging, SIMI, and fluorescence intensity to identify particles. Differentiating particles with other methodologies is more challenging since LO doesn't produce images and neither LO or FI offer SIMI or fluoresence detection.

Identification of Dynabeads in a CAR-T Cell Population

Aura CL combines BMI with FMM to distinguish between particles based on size, morphological features, and fluorescent properties. Size and morphology are useful features to differentiate between these three particles, whereas fluorescence properties definitively confirm what the particle is.

Using the scatterplot to plot particle diameter (ECD) against SIMI intensity allowed for the identification of a single population of Dynabeads (Figure 2). In combination with the grouping tool available in Particle Vue 4.0 software, we were able to account for the total Dynabeads population, CAR-T cell population and non-cellular particle population. With SIMI characterization, Dynabeads scattered more light, likely due to their higher refractive index and solid nature, whereas cells have a lower refractive index and mechanically compliant nature which will produce less light scattering when incident light is at an oblique angle.

Identifying residual Dynabead contaminants in a mixed sample was simple using Aura CL. As seen in Figure 3, cells were positive for protein (FL1) and had a greater particle diameter compared to the Dynabeads. Additionally, the scatterplot shown in Figure 4 of CAR-T cells and Dynabeads plotted as ECD (diameter) versus FL2 (Hoescht intensity) demonstrated that the cells fluoresce strongly when stained by Hoechst and Dynabeads remain non-fluorescent. Combining these characteristics (diameter, SIMI intensity and FL intensity) it is possible to easily distinguish Dynabeads from cells (Figure 5). Aura CL's high throughput analysis and built-in orthogonality enables chemical, biological, and morphological differentiation between CAR-T cells and Dynabeads, making single

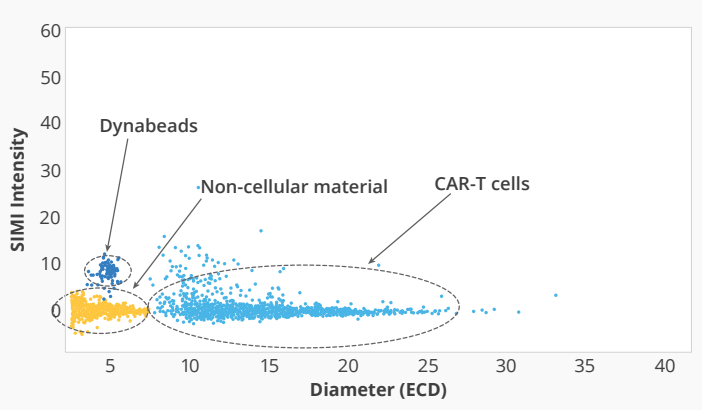

Figure 2: Scatterplot of Dynabeads mixed with CAR-T cells. In Particle Vue 4.0 software particle populations can be grouped together. By plotting diameter (X-axis) and SIMI intensity (Y-axis) Dynabeads populations (blue) can be differentiated from CAR-T cells (light blue) and non-cellular (yellow) particle populations.

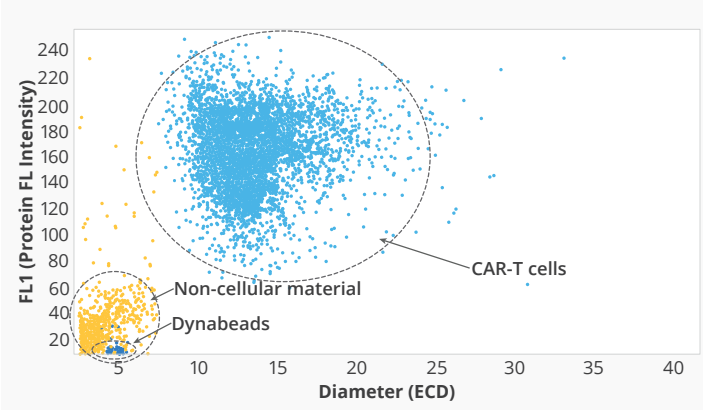

Figure 3: Scatterplot of CAR-T cells mixed with CD3/CD28 T cell Activation Dynabeads. Individual populations of Dynabeads (blue) and CAR-T cells (light blue) can be identified by their inherent physico-chemical properties following staining with a protein stain (FL1).

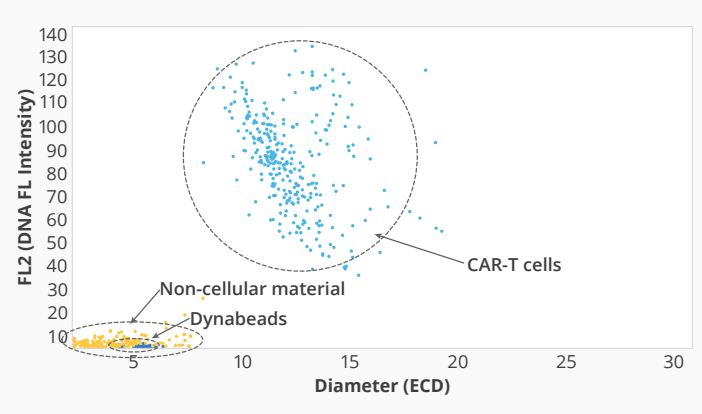

Figure 4: Scatterplot of CAR-T cells mixed with CD3/CD28 T cell Activation Dynabeads. Individual populations of Dynabeads (blue) and CAR-T cells (light blue) can be also identified by their inherent physico-chemical properties following staining with a DNA stain (FL2).

bead detection and identification easy. Aura CL achieves definitive identification and better detection of potentially dangerous product impurities and contaminants such as Dynabeads during lot analysis, validation, and release.

Discriminating between CAR-T Cells Inside a Fiber

FMM is particularly powerful when analyzing particles in close proximity. BMI only tells part of the story when particles are particularly close or stuck together. In Figure 6, the long filament observed could be characterized as one contaminant fiber using particle analysis methods with only brightfield imaging capabilities (Figure 6a). However, when you utilize the ability of Aura CL to selectively identify different types of particles with FMM, it becomes clear that the aggregate in question is not one particle, but a mixture CAR-T cells within the contaminant fiber (Figure 6b). Only the CAR-T cells within the fiber fluoresce in the FL2 channel, clearly distinguishing it from the surrounding fiber. This adds to the lack of cell counting accuracy observed with techniques that don't have the ability to clearly identify CAR-T cells from other particles in the sample using FMM.

Generating Specific Counts for the Whole Dataset using the Expression Engine Tool

The expression engine tool located in the software provides particle counts based on morphological, SIMI, and Fluorescence Intensity metrics. Through the application of Boolean logic, it is possible to provide specific particle counts for whole experiment dataset enabling rapid high-level insight and individual counts for CAR-T cells and other particles. The parameters for the expression can be determined from the scatterplot of the particles and simultaneously applied to the whole dataset (Figure 7). The expression engine tool streamlines data analysis, automatically generating quantitative data like the counts per mL for each population defined. In Figure 8, total particle counts per mL, determined by the expression engine tool, are presented and used to determine the product purity. The CAR-T sample analyzed was 69% pure. Of the 31% non-cellular contaminated detected, 64% could be attributed to the Dynabeads.

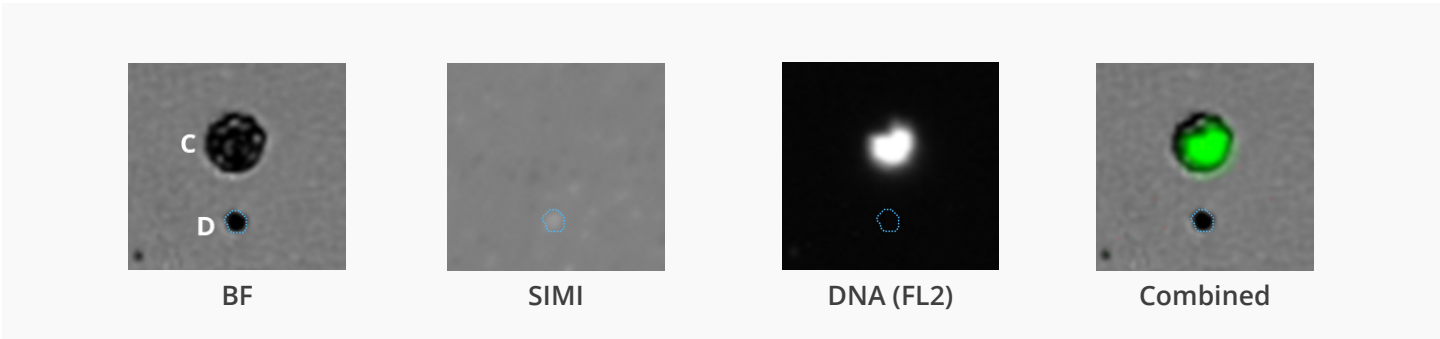

Figure 5: Dynabeads (D) and CAR-T cells (C) can be easily distinguished from one another on the basis size, SIMI intensity and DNA staining properties.

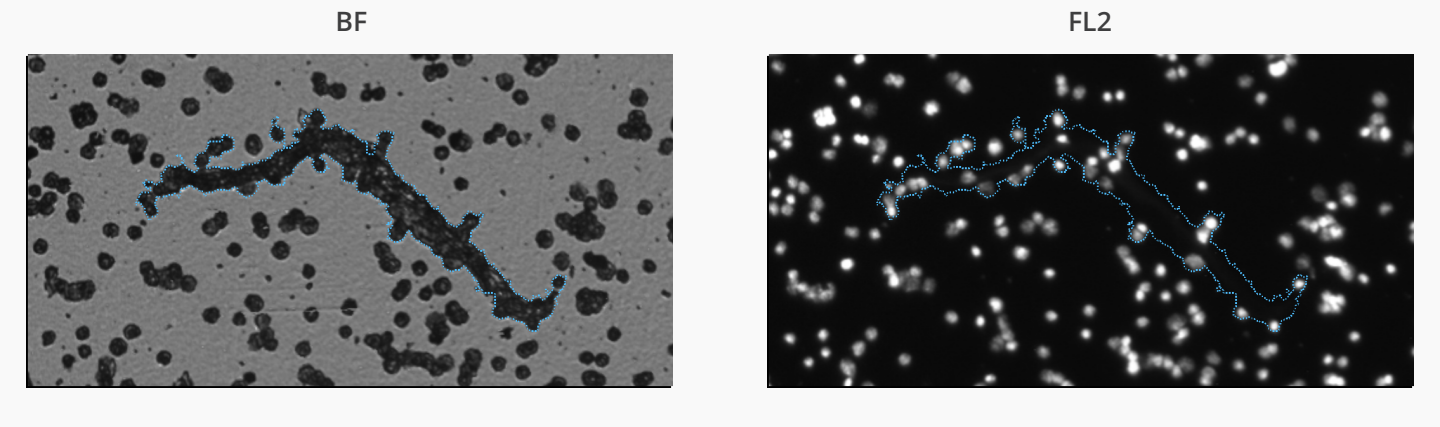

Figure 6: In a mixed population of CAR-T cells, heterogeneous aggregates can be easily identified and characterized with the combined use (a) BMI and (b) FMM imaging even when the CAR-T cells are within a fiber.

Conclusions

Aura CL is uniquely positioned to determine product purity. It fully characterize subvisible particles in cell therapy products and distinctly identifies contaminating particles such as Dynabeads and fibers introduced in the manufacturing process. Here, we showed that using BMI and SIMI in combination with FMM fluorescent stains not only allows for the rapid identification of CAR-T cells, protein aggregates, and non-biological contaminant particles, but also the identification of mixed aggregates in a single, high throughput assay. The assay is also very flexible, enabling analysis of 5 µL to 10 mL of samples, depending on your application and sample availability. With the expression engine tool available with Particle Vue software, rapid high-level insight about CAR-T cells and other particles can be captured on the whole experiment dataset and simultaneously used to accurately determine product purity. Compared to standard flow imaging

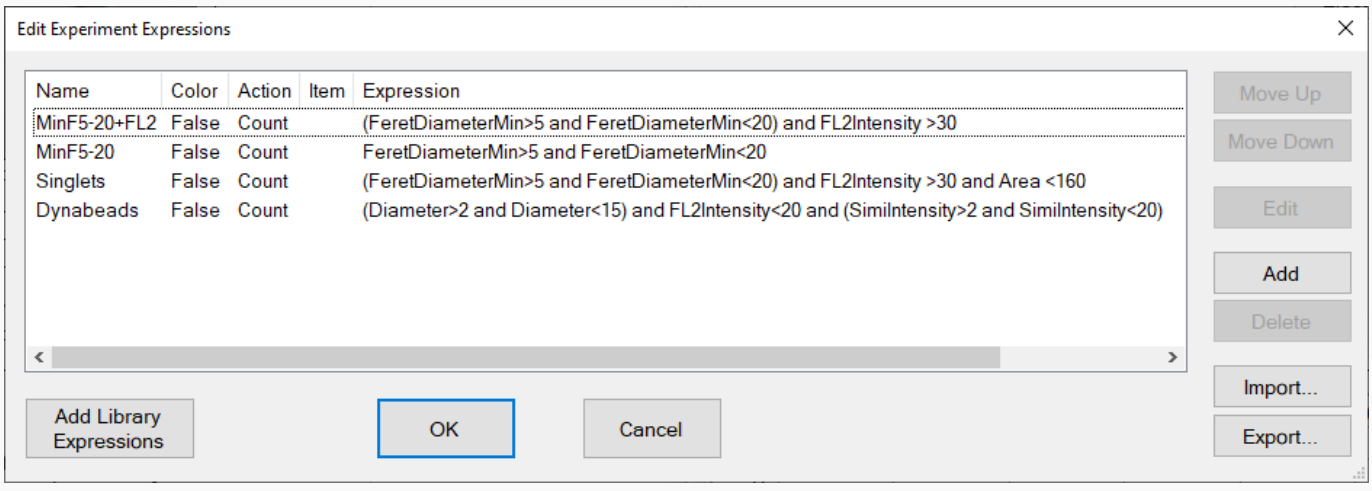

Figure 7: Boolean expressions used to identify cells and other particulates. Using the scatterplot tool, key particle parameters can be identified and used in the expression tool. For example, key metrics such as MinF 5 – 20 µm and FL2 Intensity >30 (MinF5-20+FL2) can be applied to whole dataset by using a Boolean logic within the expression tool. Here we show four expressions used to count different subpopulations of particles, CAR-T cells (MinF5-20+FL2), individual CAR-T cells (Singlets) and Dynabeads.

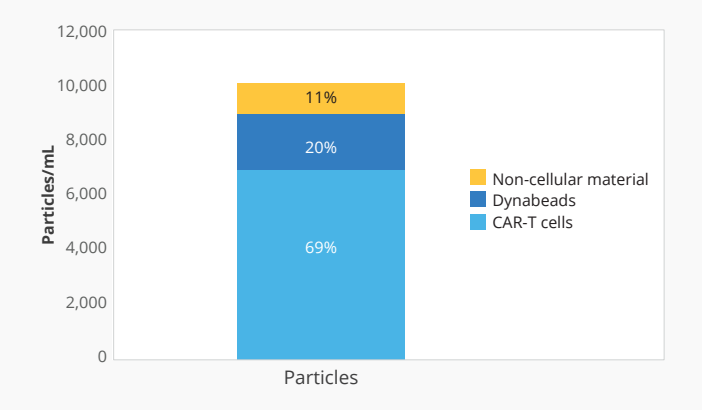

Figure 8: The expression engine tool can be applied to count an entire dataset. Here we show one dataset used to count different subpopulations of particles, individual CAR-T cells, Dynabeads, and non-cellular material contaminants.

and cytometry techniques, the throughput of FMM is 100x higher and uses best-in-class particle sizing and counting analysis that has its roots in the well-established membrane microscopy found in USP 788.

References

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