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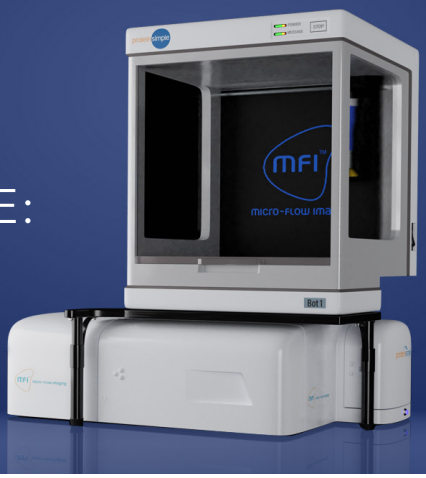
APPLICATION NOTE

ASSESS THE PURITY OF YOUR CELL THERAPY PRODUCT WITH CONFIDENCE: MICRO-FLOW IMAGING FOR NATURAL KILLER CELL THERAPEUTICS



ABSTRACT

The analysis of particle contaminants in cell therapy products is critical for ensuring drug purity and safety. However, the successful characterization of such particulates requires gaining morphological insights into each type of particle present. Analytical methods such as light obscuration and manual microscopy—while recommended for particulate analysis—provide particle count and size but are limited in their abilities to detect important information on particle morphology. To address these constraints, a technique called micro-flow imaging (MFI) enables the visualization of particulate matter by providing relevant morphological details for further study of each type of particle identified. Importantly, such details help determine contaminant-associated immunogenic risks to patients. Appropriately identifying particulates can also indicate their potential impact on drug stability and efficacy. In this study, MFI was used to distinguish between NK cells and its expansion component, Cloudz™ microspheres, in different environments. This study also shows how Cloudz microspheres were characterized with MFI, thus demonstrating the method's utility during cell expansion and formulation development.

INTRODUCTION

There are several reasons why natural killer (NK) cells are being increasingly investigated for use in cellular therapy, particularly for cancer. They express germline-encoded receptors capable of recognizing a wide variety of tumors[1]. Importantly, NK cell therapy has shown promise in clinical trials against different solid tumors[2]. NK cells also offer allogeneic capabilities that T cells do not and are less likely to cause graft versus host disease (GvHD) or cytokine storms[3,4]. Thus, efforts are geared towards reaching the goal of making "off-the-shelf" NK cell therapies.

To meet appropriate dosage requirements, NK cells need to be expanded significantly. Often, feeder cells or magnetic beads are used for this purpose, but they are not without their challenges. Magnetic particles can sometimes remain in the final product, while feeder cells pose xenogeneic challenges[5]. To address these issues, microspheres called Cloudz offer a magnet- and feeder-free platform for the expansion of immune cells. These microspheres mitigate risks associated with teratomas and are easily soluble in a Release Buffer and get washed away without the need for arduous purification processes.

It is important to confirm that the therapeutic cells do not contain impurities, whether they are product-related or process-related. According to USP <788>, injectable solutions must be checked for particulate contaminants that are larger than 10 µm and 25 µm in size. While USP <788> mentions two methods for particulate analysis—light obscuration (LO) and microscopy—it does not pose any limitations on other methods that are allowed for such testing[6]. Both LO and microscopy, while useful, are fraught with their own limitations. LO often requires high sample volumes and does not work well for turbid or viscous samples[7]. Furthermore, light obscuration does not allow the identification of different particles; it only relays particle count without providing accurate information on particle size or shape. Because the microscopic particle count test requires sample filtration before observation, some particles may undergo a change in size and shape during the filtration process, thereby appearing different under the microscope as opposed to their true shape in solution[7]. Both LO and microscopy have difficulty detecting translucent particles, are not reproducible methods, and do not provide morphological details of particles. Determining particle size,

shape, composition, translucence, etc. is important for several reasons: it helps evaluate risks posed to patients, can reveal the source of contamination, and outlines possible methods for further purification. Hence, there arises a need for an analytical method that not just provides accurate particle count, but also allows characterization of particles to obtain vital morphological details.

Micro-flow imaging (MFI) is a method designed to accurately detect, identify, and characterize the presence of sub-visible particles in a sample. These particles could be protein aggregates, cellular residues, silicone oil, glass, air bubbles, etc. The MFI 5100 system analyzes particles in the 2-300 µm range, while the MFI 5200 analyzes particles in the 1-70 µm range. In addition, a Bot1 Sampler is available to provide automated, high throughput (up to 90 samples) particle analysis.

In this study, the MFI 5200 was used to study the morphology of Cloudz microspheres in different buffers and to identify these microspheres in the presence of NK cells.

MATERIALS AND METHODS

Human Peripheral Blood CD56+ Natural Killer Cells were obtained from StemExpress (PN PB56005C) at 5 million cells per vial. GMP SCGM Medium (500 mL per bottle) was used as the cell medium, obtained from CellGenix (PN 0020802-0500). Fetal Bovine Serum (FBS) was procured from R&D Systems (PN S11110). Cells were not cultured in media; the CellGenix medium and 10% FBS were prepared for use as a diluent. Cloudz Human NK Cell Expansion Kit was obtained from R&D Systems (PN CLD004).

The Release Buffer was provided with the Cloudz Expansion Kit and was diluted to 1X. Cloudz microspheres were brought to a dilution of 1:1000 in QT-HEPES and 1X Release Buffer respectively. Cloudz microspheres were diluted at 1:100 in a mixture of the CellGenix medium and 10% FBS,. NK cells were resuspended in the CellGenix medium at 10% FBS at approximately 500,000 cells per 8 mL. Cells were counted manually using a hemocytometer. For the analysis of Cloudz microspheres in the presence of NK cells, Cloudz microspheres were added to the prepared cell suspension at a dilution of 1:100. The mixture of NK cells and Cloudz microspheres in medium were added to QT-HEPES and 1X Release Buffer separately at a 1:1 volume ratio.

All samples were analyzed on the MFI 5200 using a 100 µm Flow Cell, 1.6 mm SP3, Silane Coating (ProteinSimple, PN 4002-002-001). Prior to analysis, the instrument was calibrated with 10 µm NIST Certified Particle Size Standard (ProteinSimple, PN 4004-002-001) and with 5 µm Particle Concentration Standard (ProteinSimple, PN 4004-003-002) to ensure correct instrument operation. The volume dispensed was 0.9 mL; the sample purge volume was set to 0.2 mL. The sample volume analyzed was 0.61 mL. Optimization illumination was performed with water, and a water baseline was established before running samples. A water flush was run between samples, and after running samples containing cells, the system was flushed with 10% bleach. Data acquisition was enabled with the MFI View System Software (MVSS) version 5.1. Data processing and custom filter generation were done with the MFI Image Analysis software version 1.1.

RESULTS

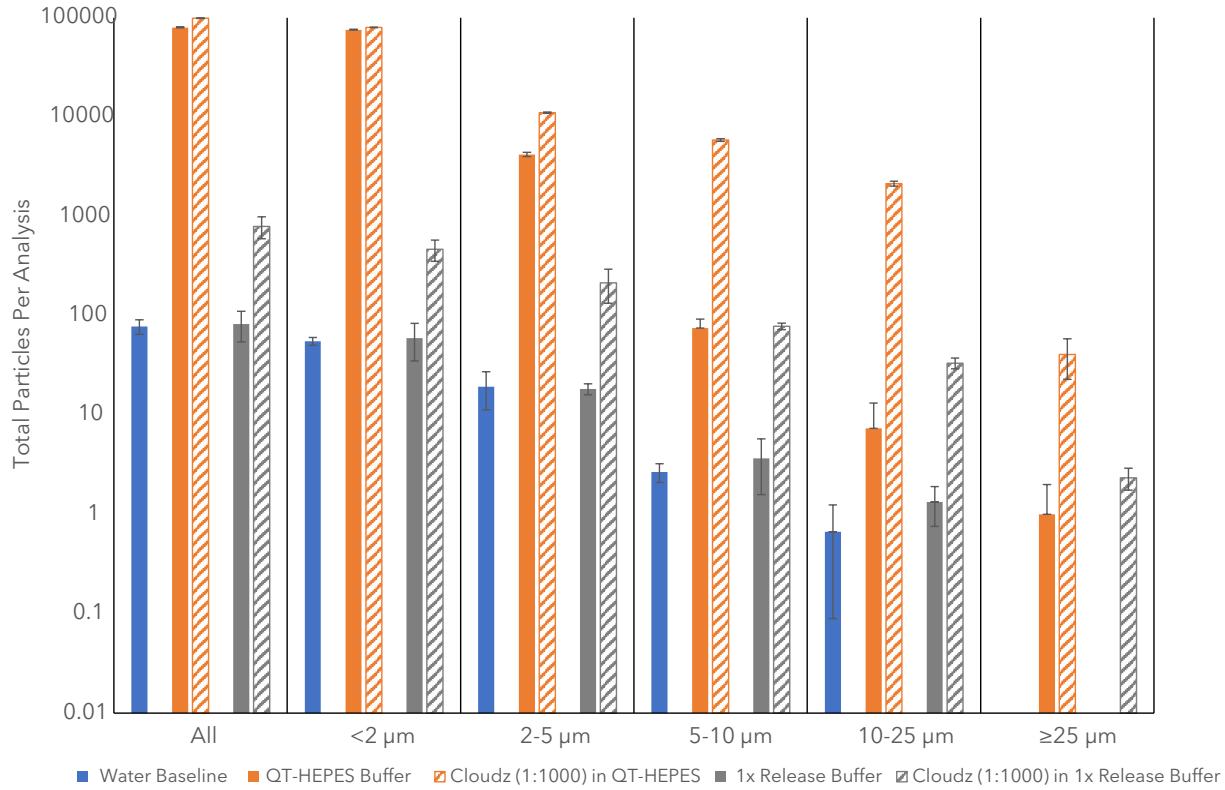
ANALYSIS OF CLOUDZ MICROSPHERES IN DIFFERENT BUFFERS

Using MFI, Cloudz microspheres were evaluated in two buffers, QT-HEPES and 1X Release Buffer, which is composed of a solution that completely dissolves the Cloudz microspheres. The two buffers were also tested without Cloudz microspheres present, and analyzed with MFI for the presence of sub-visible particulate matter. Particles observed were then grouped according to a size range, starting from less than 2 µm to greater than 25 µm. For every size group observed, QT-HEPES, with or without Cloudz microspheres, was found to contain the highest number of particles compared to the other buffers/solvents. In contrast, MFI was also able to detect a small number of particles greater than 25 µm in the 1X Release Buffer with Cloudz microspheres. This difference in particle count indicates the microspheres' dissolution in the Release Buffer, and at the same time, demonstrates that MFI is sensitive enough to detect and quantitate even a small number of particles (**FIGURES 1A and 1B**).

A

SIZE (µm)	WATER BASELINE	QT-HEPES BUFFER	CLOUDZ (1:1000) IN QT-HEPES	1X RELEASE BUFFER	CLOUDZ (1:1000 IN 1X) RELEASE BUFFER
	TOTAL PARTICLES PER ANALYSIS				
ALL	78.0	79457.7	98861.0	82.7	793.3
<2	55.3	75185.0	79761.0	59.3	465.3
2-5	19.3	4189.3	11045.7	18.3	214.0
5-10	2.7	75.0	5875.0	3.7	78.3
10-25	0.7	7.3	2138.7	1.3	33.3
≥25	0.0	1.0	40.7	0.0	2.3

B



Error Bars = STD DEV (N=3)

FIGURE 1. MFI Analysis of Particles in Different Buffers/Solvents. A. Particle counts (total particles per analysis) in three distinct solvents: water (**blue**), QT-HEPES (**orange**), and the 1X Release Buffer (**grey**). QT-HEPES and 1X Release Buffer were analyzed with and without the presence Cloudz microspheres. All particulate matter detected were categorized based on size ranges. QT-HEPES buffer-with and without Cloudz microspheres—was found to have the highest number of particles among the three solvents. **B.** A bar graph representing the total number of particles per analysis in each buffer or solvent. The particle count per buffer is grouped according to the size of particles observed. Error bars represent standard deviations.

GATHERING MORPHOLOGICAL INFORMATION OF DETECTED PARTICLES

The characterization of different particles was conducted with the MFI Image Analysis Software, which allows the creation of custom filters around morphological parameters to identify and classify different particle types. The filters applied were selected from 10 different morphological parameters such as circularity, equivalent circular diameter (ECD), aspect ratio etc. A complete list of applicable morphological parameters in the software is shown in **FIGURE 2**.

MORPHOLOGICAL PARAMETERS AVAILABLE IN MFI IMAGE ANALYSIS SOFTWARE

- Equivalent circular diameter (ECD
- Area
- Perimeter
- Circularity
- Aspect ratio
- Max Feret diameter
- X position
- Intensity mean
- Intensity standard deviation
- Intensity minimum
- Intensity maximum
- Time (%)
- Time (minutes)

FIGURE 2. The 10 different morphological parameters available on the MFI Image Analysis software, based on which filters can be applied for detailed particle analysis.

Using MFI Image Analysis software, Cloudz microspheres were identified and isolated from other particulates detected in the different solvents/buffers used. Applying stringent custom filters for Cloudz microspheres uncovered a remarkably high number of intact Cloudz microspheres in QT-HEPES, in comparison to the 1X Release Buffer (**FIGURES 3A and 3B**). This is consistent with the results shown in **FIGURE 1**, where QT-HEPES is shown to have the highest particle count. Further analysis revealed that most of the Cloudz particles in QT-HEPES were 4-12 µm in size, whereas a relatively smaller number of Cloudz particles were found to be greater than 18 µm, as shown in **FIGURE 4**.

A

Name	Property	Test	Level
All	Remove Edge, St...		
Filter1	Circularity	>=	0.9
Filter2	Aspect Ratio	>=	0.9
Filter3	ECD	>=	4.0
Cloudz	Intensity Min	>=	140.0

B

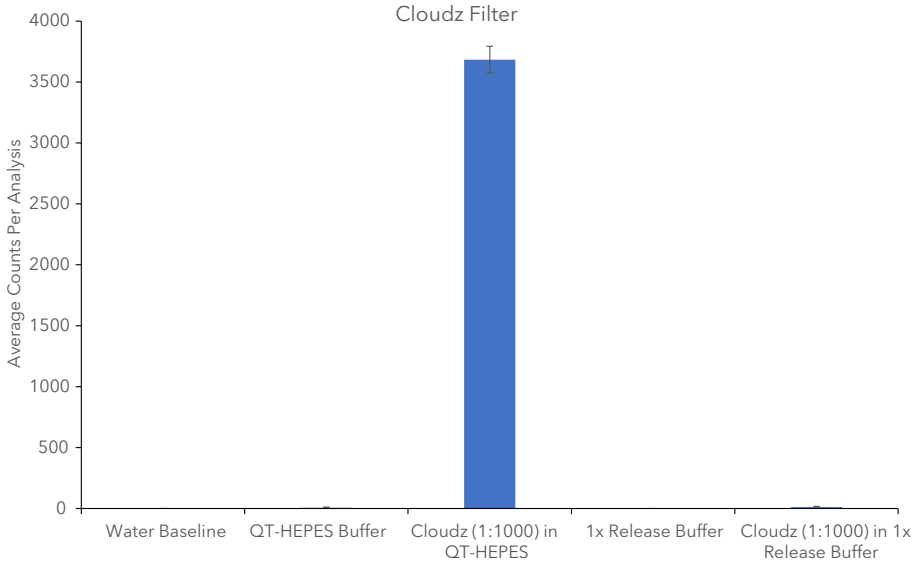


FIGURE 3. Analysis of a specific group of particles using the MFI Image Analysis Software. A. Based on four different morphological characteristics of Cloudz microspheres, custom filters were applied to determine their count amongst other particles. **B.** The bar graph represents the average counts of Cloudz microspheres per analysis in different solvents. QT-HEPES shows the highest count of Cloudz microspheres. Error bars represent the standard deviation.

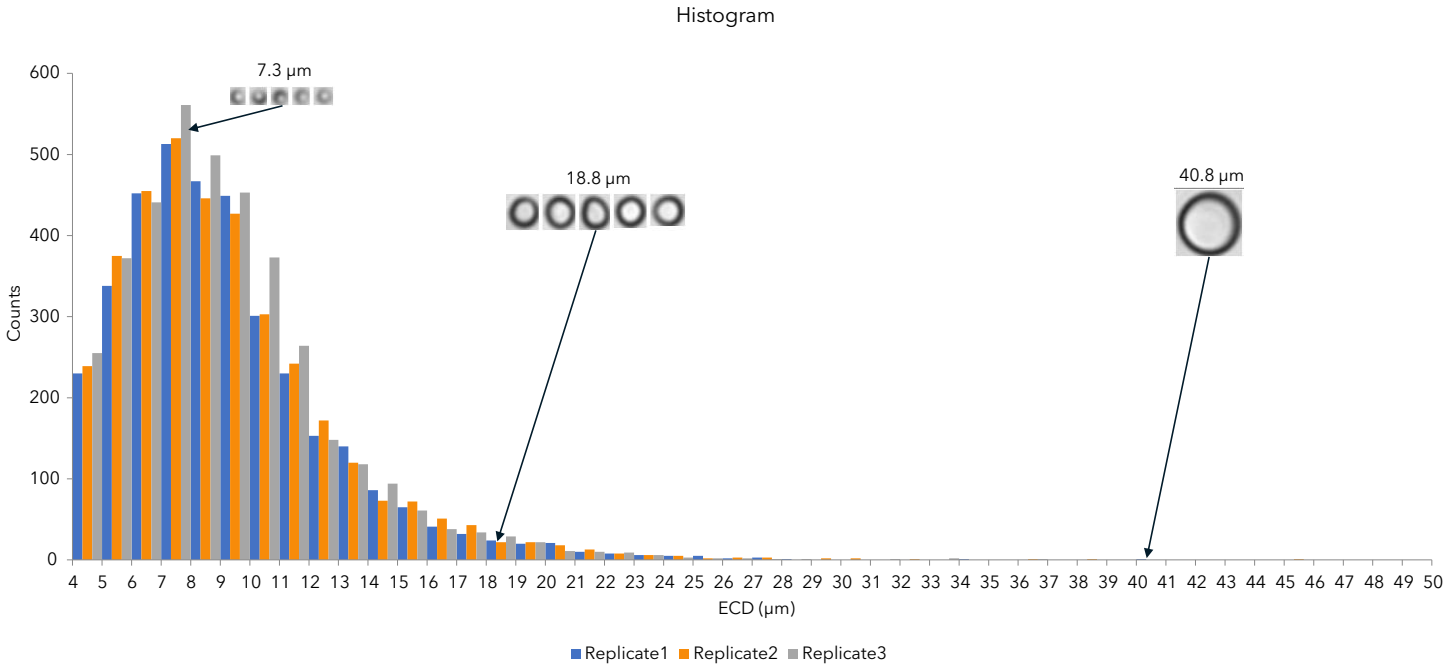


FIGURE 4. Size distribution of Cloudz microspheres in QT-HEPES. The filters applied in the MFI Image Analysis software showed that 4-12 µm was the size range for the majority of filtered Cloudz microspheres.

As the role of the Release Buffer is to dissolve Cloudz microspheres and make their removal easy after NK cell expansion, MFI was next used for the analysis of these microspheres dissolved in 1X Release Buffer. Based on the size of Cloudz microspheres determined from previous experiments, filters were applied to detect their presence. As expected, analysis by MFI showed no detectable Cloudz microspheres in solution, thus indicating that they completely dissolved in the Release Buffer (**FIGURE 5**).

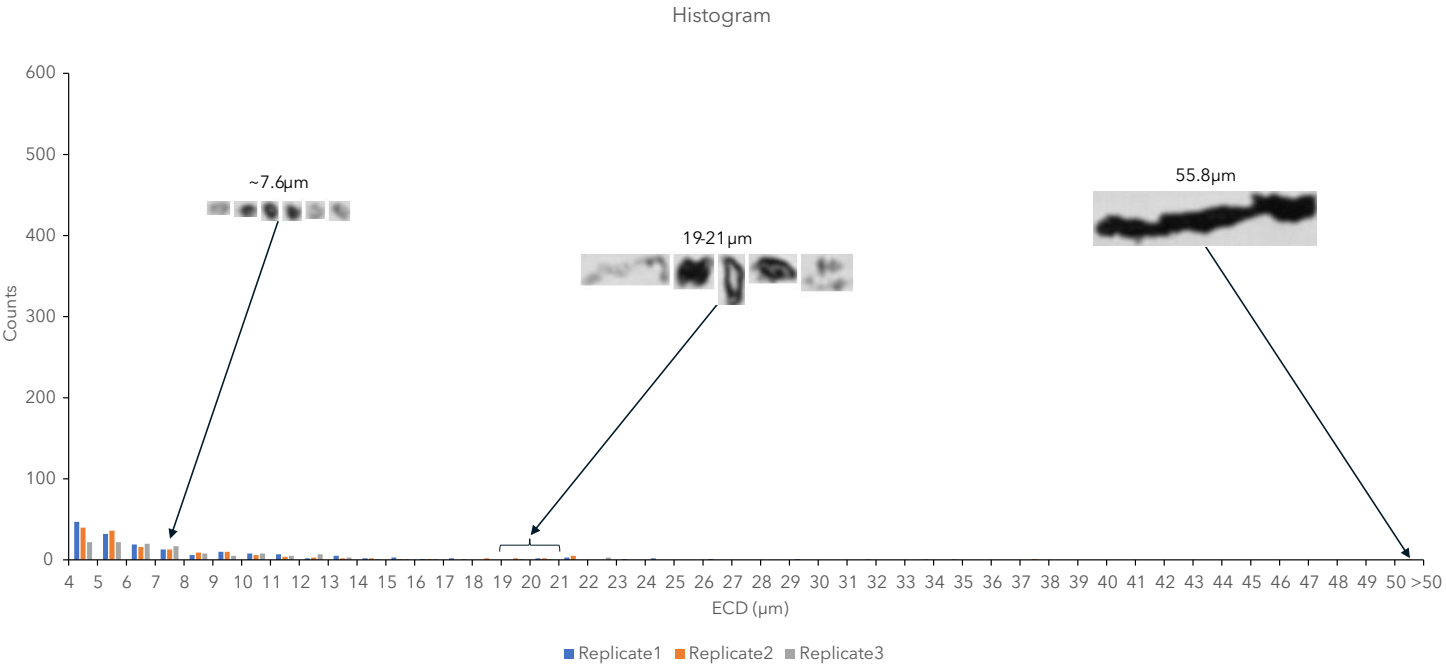


FIGURE 5. Detection of Cloudz microspheres in 1X Release Buffer. An ECD filter of ≥ 4 µm was applied across triplicate injections to detect Cloudz microspheres in 1X Release Buffer. Upon analysis, no distinct population of Cloudz microspheres was observed.

In addition to QT-HEPES and the Release Buffer, the presence of Cloudz microspheres was evaluated in other reagents used in cell culture and expansion. Interestingly, their morphology changed depending on the buffer they were in, particularly in the CellGenix medium. As described previously, the microspheres were easily distinguishable in QT-HEPES, however, they embodied a distinct swollen structure in the CellGenix medium mixed with 10% FBS (**FIGURE 6, panel B**). Finally, with both NK cells and Cloudz microspheres in a mixture of QT-HEPES and medium, MFI was able to identify the microspheres, as shown in **FIGURE 6**, last panel from the left.

To further test the behavior of Cloudz microspheres in the cell medium, varying volumes of the 1X Release Buffer were mixed with the medium. Notably, the microspheres required a 1:1 mixture of the 1X Release Buffer and CellGenix medium to fully dissolve. No Cloudz microspheres were observed at this dilution (data not shown).

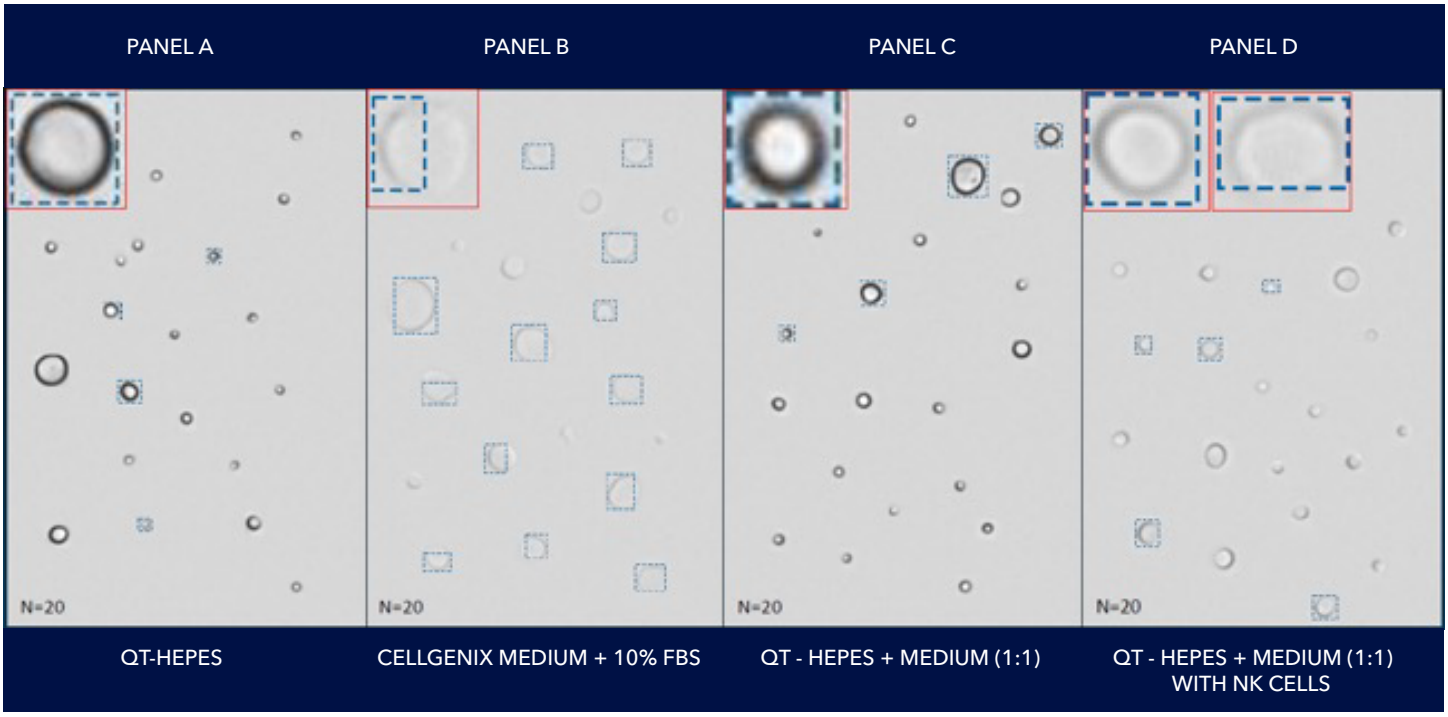


FIGURE 6. Cloudz particles demonstrate differences in morphology depending on the buffer used. Images from MFI clearly show the swollen Cloudz structure in the presence of CellGenix Medium. Images enclosed in red were not scaled.

MFI was next used to identify Cloudz microspheres in the presence of NK cells in different reagents. In the CellGenix medium, both NK cells and Cloudz particles were detected. A similar observation was made when QT-HEPES buffer was added to the medium with NK cells and Cloudz particles. In contrast, when a mixture of NK cells and Cloudz particles was treated with a mixture of CellGenix medium and 1X Release Buffer, Cloudz particles were nearly undetectable, indicating that the Release Buffer had dissolved the microspheres, as expected. (**FIGURE 7, inset bar graph**).

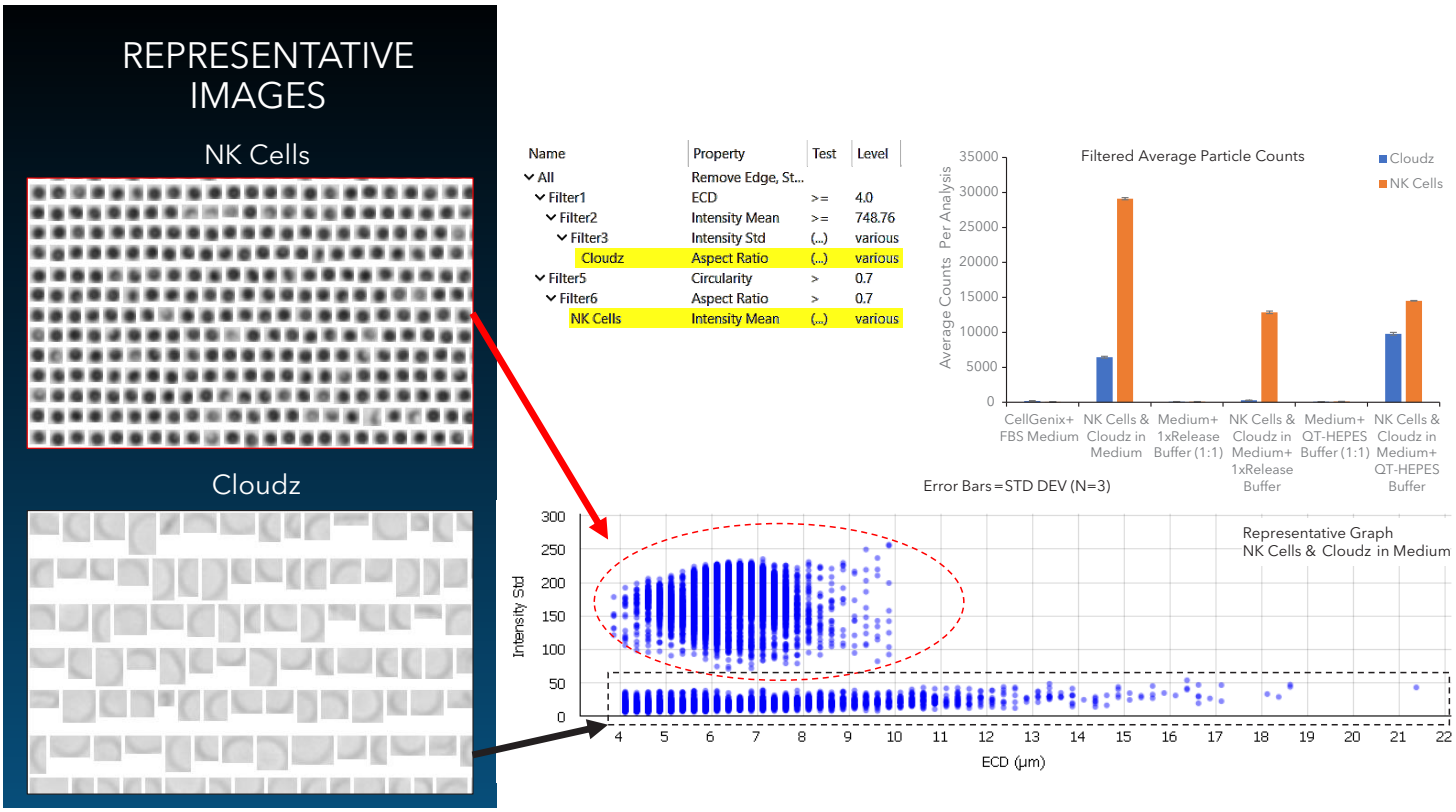


FIGURE 7. Distinguishing between NK cells and Cloudz microspheres with MFI. When NK cells and Cloudz particles were mixed together in different buffers, custom filters were applied to look specifically for NK cells and Cloudz particles. Depending on the buffer used, a different particle count was reported for both.

CONCLUSION

The expanding applications of NK cell therapy – owing to its potential for broader applications and allogeneic promise – demands reliable analytical tools that can test for different CQAs of therapeutics. During the determination of sample or product purity, the analysis of visible and sub-visible particulate matter is crucial. Traditional methods such as light obscuration and light microscopy provide information on particle count and size distribution, but do not allow further characterization of particles for their specific size, shape, translucence etc. Having this information is critical for categorizing particles, identifying sources of contamination, assessing their immunogenic potential, and determining next steps for further purification.

Consequently, counting particles, distinguishing them from one another, and gathering relevant morphological information with confidence is made possible using MFI. This study has shown that MFI, with its direct imaging capabilities and powerful software tools, can clearly differentiate between NK cells and Cloudz microspheres. Analysis with MFI revealed that particle counts and morphology of the Cloudz microspheres varied among buffers, indicating key differences in solubility. As such, MFI is a powerful tool that can provide crucial insight into even small numbers of subvisible particles within a cell therapy product.

For more information on how MFI can be applied to cell therapy development, read our application note on determining residual bead count during CART-cell manufacturing.

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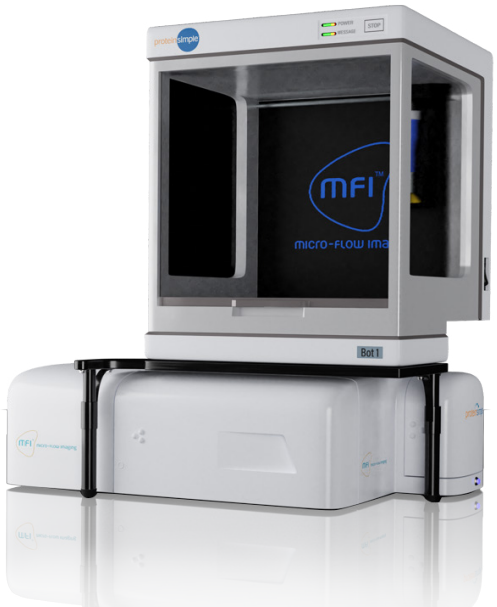

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