FlowCam®

Measuring Subvisible Particles and Aggregates using FlowCam LO

SUMMARY

Measuring subvisible particles in pharmaceutical products is of importance to manufacturers and regulatory agencies to ensure product quality, stability, and efficacy. For years, pharmacopeial methods have relied on light obscuration (LO) as the primary means for detecting particles in the 2-100 µm range[1]. With the growth of protein-based therapeutics, viral vectors and nano-drug delivery systems, additional consideration has been given to particles formed by aggregation, as numerous publications have identified aggregation as a high-risk factor for adverse immunogenic reactions[2,3,4,5]. Since these aggregates are often highly transparent, LO is inherently less useful as a technique for effectively measuring and characterizing these particles. Flow imaging microscopy (FIM) has emerged as an essential tool in the characterization and identification of protein aggregation due to its higher sensitivity to highly transparent particles[6]. FIM also records images of each particle that can be used to distinguish different types of aggregates and other particle types in a sample.

Recently, FlowCam LO has been introduced to allow users to simultaneously collect FIM and LO data from a single aliquot of sample. Here we use FlowCam LO to directly compare the particle size distributions of aqueous samples containing Polystyrene Latex calibration beads, ETFE particles, and IgG aggregates obtained from both LO and FIM analyses. These results highlight how a combined LO-FIM approach, using FlowCam LO, can help researchers accurately detect, count, and analyze particles in biotherapeutic samples while still meeting pharmacopeial requirements for particle monitoring.

INTRODUCTION

As biologics have grown in use and complexity, LO has shown to be insufficient as an exclusive tool for subvisible particle characterization. A major limitation of light obscuration is its inability to differentiate particle types (e.g., protein aggregates, silicone oil microdroplets, extrinsic particles) that may be encountered in these samples. LO is also less effective at analyzing high protein concentration formulations due to the higher turbidity, viscosity, and particle concentrations (i.e., coincidence rates) of these concentrated therapeutics[7]. Even more problematic is the limited sensitivity of LO to highly transparent particles like those commonly found in formulations of proteins[8], viral vectors, and most drug delivery systems. The limited sensitivity of LO to these highly transparent particles may result in artificially low particle counts.

To address these deficiencies, the biopharmaceutical industry has widely adopted flow imaging microscopy as a powerful orthogonal method of subvisible particle characterization to improve product quality, stability, and efficacy. This modality has become the industry norm despite not having previous directives from regulatory agencies. However, following the revision of USP <1788> *Subvisible Particulate Matter* in May 2021, the US Food and Drug Administration (FDA) officially recognizes FIM as a complimentary, orthogonal method to LO, validating the need for more rigorous interrogation of proteinaceous aggregates than is possible with LO alone.

The clear advantages of FIM and the recent shift in the pharmacopeial guidance has created the need for a simple, comprehensive solution for characterizing subvisible particles and aggregates. FlowCam LO is the first and only LO instrument with imaging capabilities. Combining FIM with LO delivers a powerful, all-in-one solution that allows users to simultaneously collect the LO data necessary to meet compliance standards as well as the imaging data useful in detecting translucent particles and differentiating between different particle types in a sample.

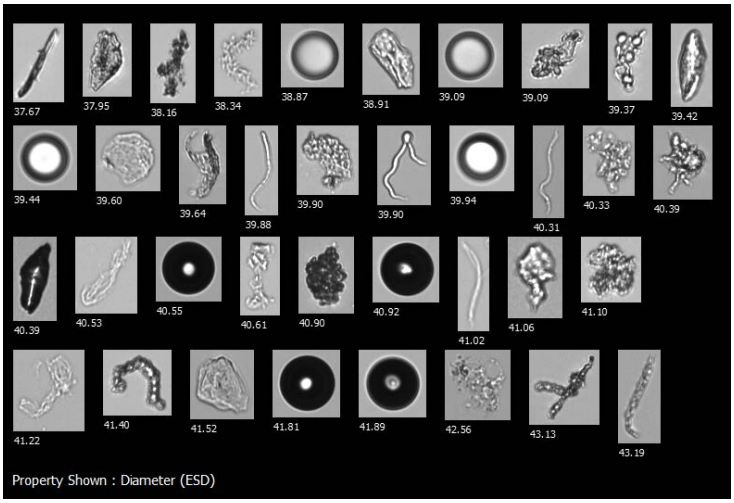

Figure 1. Sample FIM images of particle types commonly found in biotherapeutics including protein aggregates, silicone oil microdroplets, and air bubbles

The imaging data can be especially useful for analyzing heterogeneous particle populations like those commonly found in biotherapeutics. Figure 1 shows sample FIM images of particles 35-45 µm in diameter. While LO would not be able to distinguish these particles, the FIM images can be used to differentiate between protein aggregates, silicone oil droplets, air bubbles, and fibrils in this sample. FlowCam LO gives the user the data needed to be certain about the types of particles present in a biotherapeutic sample.

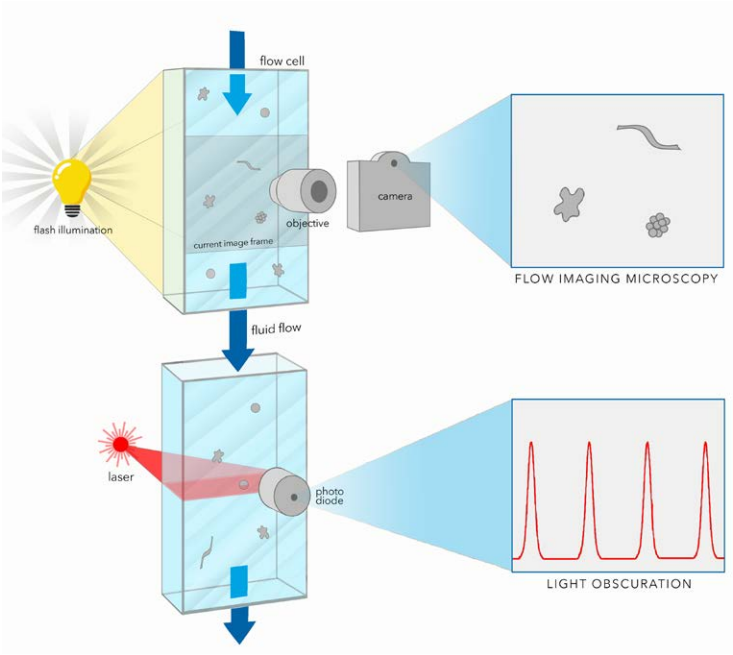

Figure 2. FlowCam LO schematic showing how a liquid sample is analyzed by the instrument. Fluid flows through an FIM module (top), followed by an LO module (bottom), yielding particle measurements by both modalities.

FlowCam LO follows a serial measurement approach in a single fluidics flow path. Particles are first imaged using FIM and then pass through the LO module as shown in Figure 2. VisualSpreadsheet® software captures both data sets simultaneously, allowing the user to analyze the results and generate reports using a single software package.

This design allows the user to collect both LO and FIM data with a single aliquot of sample, reducing the same volume and time required to obtain both measurements. Additionally, as each modality records measurements of the same sample, FlowCam LO also allows the user to directly compare the performance and results from these two orthogonal techniques.

MEASUREMENTS & RESULTS

To illustrate the benefits of LO with imaging, samples containing three particle types were prepared and analyzed with FlowCam LO: Polystyrene Latex calibration beads, abraded ethylene-tetrafluoroethylene (EFTE) and IgG aggregates. All three samples were analyzed using the same settings on the instrument. 1 mL aliquots of each sample were analyzed at a flow rate of 0.2 mL/min. Particle segmentation via FIM was performed using 18 dark pixel and 20 light pixel thresholds and two close hole iterations. These settings were used to increase the sensitivity of the particle segmentation which we anticipated would improve the detection of translucent particles via FIM at the cost of some slight particle oversizing.

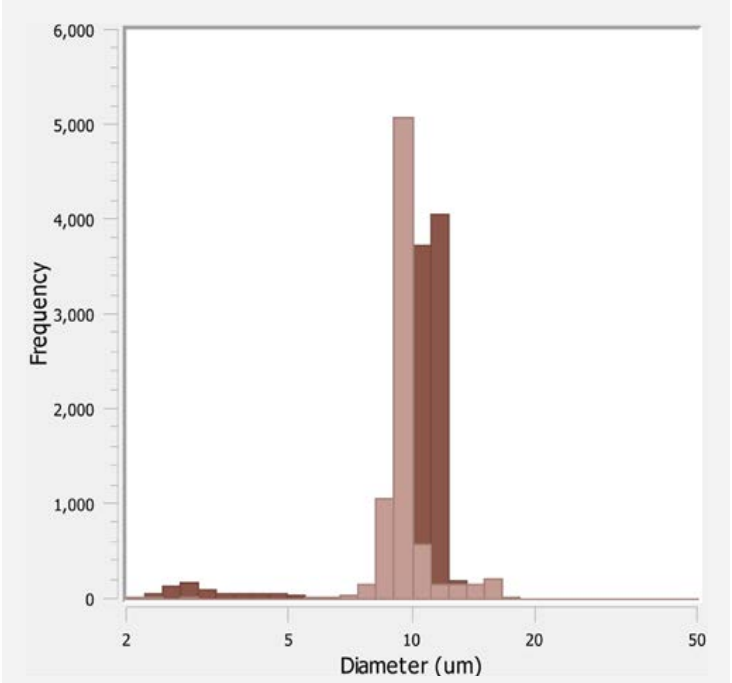

Figure 3. Particle size distribution of 10 µm calibration beads obtained from FlowCam LO as returned by VisualSpreadsheet. The vertical axis shows the number of particles assigned to each size bin. Light bars correspond to particle diameters measured by LO, dark bars correspond to particle diameters measured by FIM. Approximately 2,000 particles imaged via FIM are in the same size bin as the bin containing most of particles for LO but are obscured due to the histogram stacking.

10 µm calibration beads were analyzed using FlowCam LO to compare the performance of the FIM and LO modules for detecting and sizing high contrast particles. Figure 3 shows the particle size distributions obtained from both FIM and LO. The two modalities yielded reasonable agreement in particle counting and particle sizing relative to the nominal bead size, suggesting that both the LO and FIM modules in FlowCam LO counted and sized beads correctly. The higher diameters reported by FIM for the calibration beads (10.6 µm on average for in-focus beads) were likely a consequence of the particle segmentation settings on the FIM module. More stringent particle detection settings on FIM may improve the agreement between the sizes reported by the two modalities.

ETFE is a NIST recognized surrogate protein particle standard with several desirable properties: it has a refractive index close to that of amorphous protein and, when abraded, the morphology of the resulting particles closely mimics that of typical protein aggregates.

Figure 4 shows sample particle size distributions obtained for an ETFE particle solution from both the FIM and LO modules in FlowCam LO as generated by the VisualSpreadsheet software. FIM identified an overall higher number of particles than LO, especially in the 2-5 µm diameter range, suggesting that LO missed highly transparent particles that FIM more readily identified. Fig. 4 also suggests that LO undersized many of these translucent particles. While both size distributions exhibited a particle population larger than 10 µm, these particles were assigned a lower diameter by LO (10-20 µm) than by FIM (20-30 µm). As a result, many particles that were sized between 10-25 µm via LO were larger than 25 µm when sized via FIM—a size range with more stringent count limitations per USP <788> than those between 10 and 25 µm.

IgG samples from human serum were prepared in PBS buffer solution and vortexed to induce aggregation. Figure 5 shows sample FIM images and Figure 6 shows the particle size distributions of these aggregates measured by FlowCam LO and generated by VisualSpreadsheet. As can be seen in the images, IgG aggregates generated via vortexing exhibited very high transparency. This transparency resulted in drastically reduced particle counts when measured by LO relative to those measured by FIM. While this represents an extreme example, these results highlight the poor sensitivity of LO to highly transparent particles that are much more readily detected by FIM.

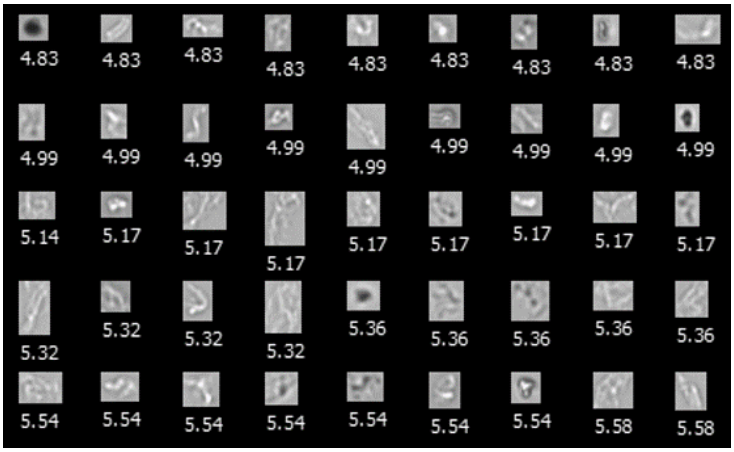

Figure 5: Sample FIM images of IgG aggregates ~5 µm in diameter

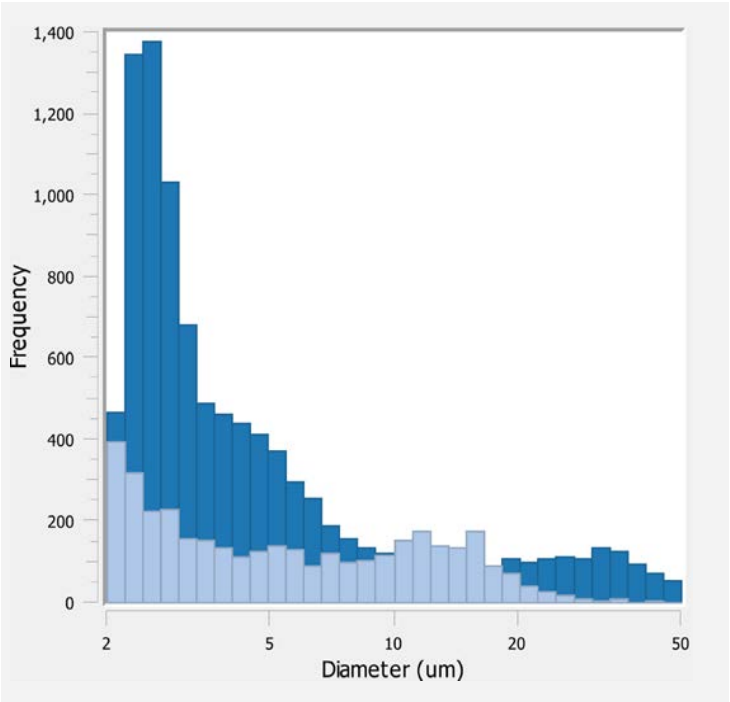

Figure 4: Particle size distribution of ETFE particles obtained from FlowCam LO as returned by VisualSpreadsheet. The format of this figure matches that of Figure 3.

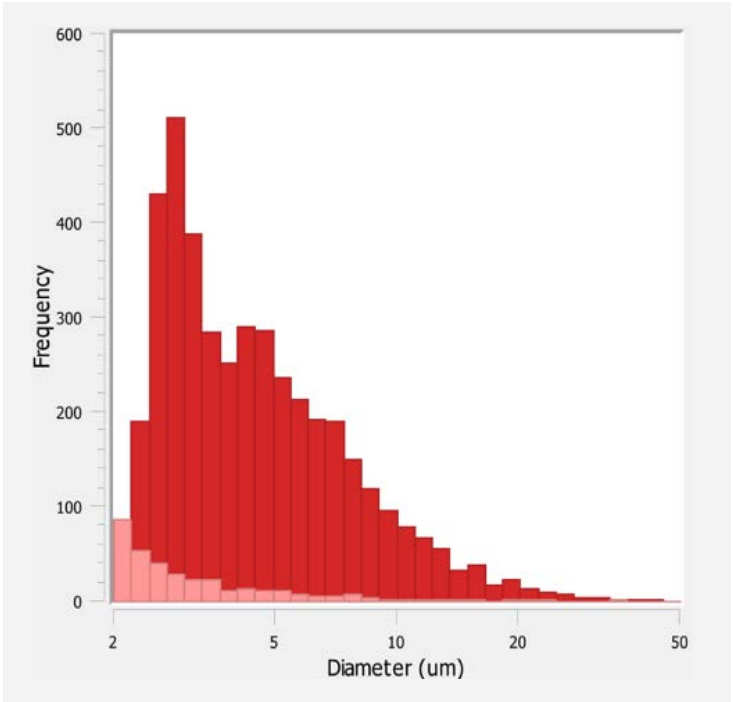

Figure 6: Particle size distribution of IgG aggregates obtained from FlowCam LO as returned by VisualSpreadsheet. The format of this figure matches that of Figure 3.

CONCLUSIONS

Light obscuration (LO) is a widely used subvisible particle assay in the pharmaceutical industry and is cited in compendial methods (USP, EP, JP). Despite its prevalence, LO's limited sensitivity to translucent particles and inability to differentiate between particle types often hinders the technique relative to orthogonal techniques like flow imaging microscopy (FIM). The results presented here, utilizing FlowCam LO to obtain simultaneous FIM and LO measurements of a single sample, demonstrate that LO can undersize or even completely fail to detect highly transparent particles such as ETFE and some protein aggregates that FIM easily detects.

These results also illustrate how FIM images can be useful in differentiating between particle types—including particle types of the same size that would not be differentiated by LO. Most importantly, these results highlight the importance of pairing LO measurements with an orthogonal technique like FIM to effectively count and size translucent particles and to extract particle type information that is not accessible with LO alone. FlowCam LO greatly simplifies collecting these orthogonal measurements by including both modalities on a single, easy to use instrument, allowing the user to quickly and easily extract more information about the particles in their biopharmaceuticals.

REFERENCES

1. USP/NF Monograph <788>. Particulate matter in injections. In. Rockville, MD: United States Pharmacopeial Convention; 2014.

2. Rosenberg AS. 2006. Effect of protein aggregates: An immunologic perspective, AAPS J 8:E501-E507.

3. Rosenberg AS, Verthelyi D, Cherney BW. Managing uncertainty: a perspective on risk pertaining to product quality attributes as they bear on immunogenicity of therapeutic proteins. J Pharm Sci. 2012;101(10):3560–7.

4. Filipe V, Jiskoot W, Basmeleh AH, Halim A, Schellekens H, Brinks V. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. mAbs. 2012;4(6): 740–52.

5. Bi V, Jawa V, Joubert MK, Kaliyaperumal A, Eakin C, Richmond K, et al. Development of a human antibody tolerant mouse model to assess the immunogenicity risk due to aggregated biotherapeutics. J Pharm Sci. 2013;102(10):3545–55.

6. Sharma DK, Oma P, Pollo MJ, Sukumar M. Quantification and characterization of subvisible proteinaceous particles in opalescent mAb formulations using micro-flow imaging. J Pharm Sci. 2010;99(6):2628–42.

7. Singh S, Afonina N, Awwad M, Bechtold-Peters K, et al. An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J Pharm Sci. 2010;99(8):3302-3321.

8. Zölls S, Gregoritza M, Tantipolphan R, Wiggenhorn M, Winter G, Friess W, Hawe A. How subvisible particles become invisible-relevance of the refractive index for protein particle analysis. J Pharm Sci. 2013;102(5):1434-46