APPLICATION NOTE

Particle analysis of therapeutic protein formulations with ImageStreamX® Imaging Flow Cytometry and the ProteoStat® Protein Aggregation Assay

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Abstract

The formation of protein aggregates (PA) and other subvisible particles within protein-based drugs is a major concern for the pharmaceutical industry because they can impact drug activity or cause unwanted immunogenicity in patients (1). It is critical to monitor the presence of these particulates to ensure the safety and efficacy of these drugs, especially since their formation can be highly sensitive to production and storage conditions. Currently US and European pharmaceutical regulations require testing of subvisible (>10 um) and visible (>25 um) particles of therapeutics, and recent literature has highlighted the importance of also measuring particles in the 1-10 um size range (2). Another emerging requirement is the need to classify particle type, as multiple contaminants including silicone oil droplets and bacteria may be present in this size range (3). No currently accepted industry method meets the need to simultaneously size, enumerate, and classify particles within this critical 1-100 um size range, thus illustrating the need to develop new approaches for this application (3). In this study we evaluate the potential for the Amnis ImageStreamX MKII® (ISX) imaging flow cytometer to address these criteria and enable comprehensive particulate characterization for therapeutic protein formulations. The major advantage of the ISX is the coupling of flow cytometry with microscopy, which allows particle type to be classified using specific fluorescence stains and quantitative measurement of morphological properties such as size from high resolution imagery. Though traditional flow cytometry has been previously applied for this application (4), it is limited compared to imaging flow cytometry because it cannot directly size particles, is less sensitive for detection of small and translucent particles, and does not measure other potentially important morphological parameters such as shape and contrast. In this study we used Enzo Life Sciences ProteoStat® Protein Aggregation Standards as a model PA system. ProteoStat® Protein aggregation Standards are the only commercially available PA standards, and provide a useful tool for measuring the linearity and sensitivity of the ISX for PA analysis. Additionally, the ProteoStat® Protein Aggregation assay was used to fluorescently label PAs with high specificity. This simple mix-and-read assay offers high selectivity for PAs using a proprietary fluorescent (FL) probe that displays 20-90 fold brightness enhancement upon binding to the cross-beta spine quaternary structure of aggregated proteins. As silicone oil microdroplets are a common contaminant for PA applications, we further evaluated the ability of ISX to differentiate this contaminant using Bodipy fluorescent dye. Through this study we determined that the ISX offers multiple advantages for PA analysis compared to currently accepted platforms, including (i.) direct measurement of particle size and other morphological characteristics through quantitative image analysis (ii.) classification of multiple particle types by incorporating additional fluorescent stains, (iii.) collection of statistically robust data sets via rapid image acquisition, (iv.) measurement of absolute particle concentrations using a volumetric syringe pump, and (v.) small minimum sample volume requirement of 20 µL.

Materials and Methods:

Sample preparation:

IgG only samples

ProteoStat® Protein Aggregation Standards (ENZ-51039) were reconstituted in filtered deionized water to final concentrations of 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.20%, and 0% aggregated IgG (total protein concentration fixed at 1 mg/mL). Samples were fluorescently labeled using the ProteoStat® Protein Aggregation Assay (ENZ-51023) following the protocol provided by the manufacturer.

Mixed IgG and silicone oil samples

A solution containing silicone oil microdroplets (SiOil) was prepared by mixing 100 µL silicone oil with 50 mL of water and vortexing vigorously for one minute. PA/SiOil mixed samples were prepared by mixing equal volumes of SiOil and 0.39% PA solutions. Samples were incubated with ProteoStat® Detection Reagent and Bodipy dye (final concentration 1 um) for 15 minutes to fluorescently label PAs and SiOil respectively.

Flow cytometry

Flow cytometry was performed with a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin lakes, NJ, USA). The data was collected with BD CellQuest software andprocessed with FlowJo 7.6.3 software (Tree Star, Ashland, OR, USA). The samples were analyzed with high flow rate and PMT voltages were adjusted for the size range of Size Standard from 1 µm to 10 µm. The measurements were stopped after 5000 events were counted. The events were detected by FL1 for Bodipy dye and FL3 for ProteoStat® dye. The size of the detected aggregates was estimated by comparing the amount of side scatter with the amount of side scatter produced by the known size standards.

Data acquisition and analysis:

The ImageStreamX-MKII® was used to collect imaging flow cytometry data for each sample. Magnification was set at 60x (0.33 um pixel resolution). A 488 nm excitation laser (200 mW) was used to excite Bodipy and ProteoStat fluorescence. Brightfield (BF) imagery was collected using an LED-based BF illuminator. A dedicated 785 nm laser (20 mW) was used to collect side scatter (SSC) measurements. The number of events per sample was 50,000 for IgG only experiment and 2,000 for mixed IgG and SiOil experiment. Image analysis was completed using quantitative image algorithms available in the IDEAS 6.0 image analysis software package. SpeedBead® Calibration reagent events were removed from analysis using forward-side scatter discrimination, and clipped objects were eliminated using the Raw Centroid X feature. Volumetric percent was calculated by multiplying particle concentration by mean BF particle diameter using the appropriate unit conversion. A fluorescence plate reader (Infinite 200 Pro-Tecan) was used to measure absolute sample fluorescence.

Figure 1: ImageStreamX MKII ® Instrument Configuration

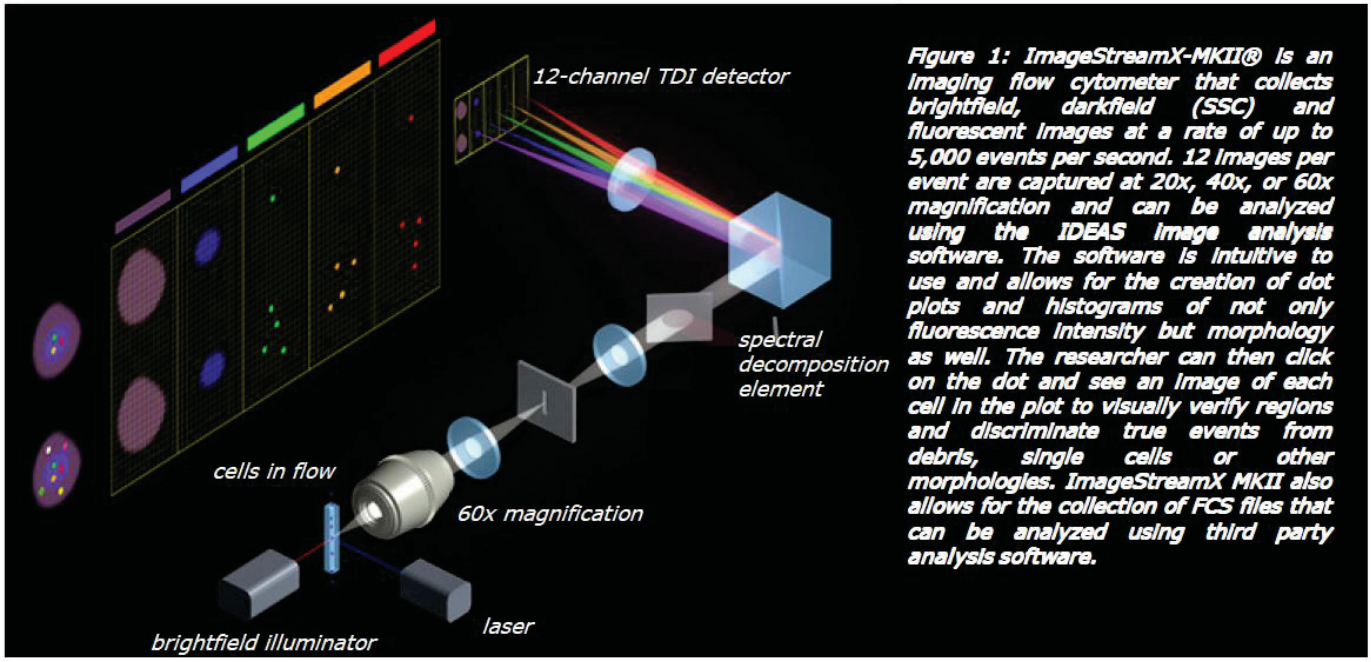
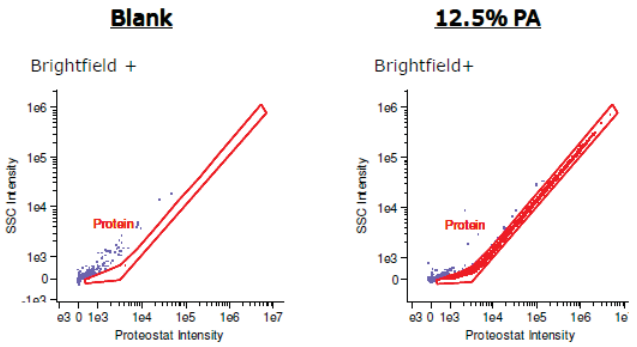
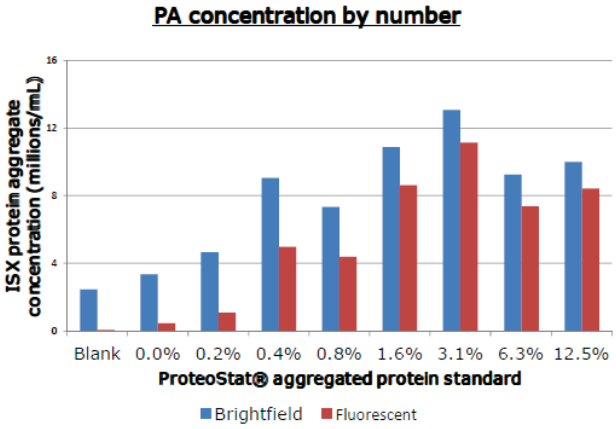


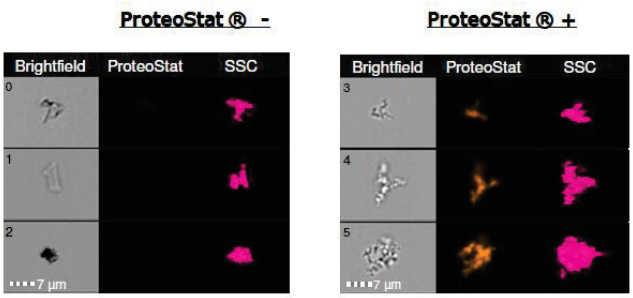
Figure 2: Protein aggregate quantification using ProteoStat® detection reagent compared to Brightfield detection.



A. Bivariate intensity plots for ProteoStat® vs. SSC channels using the BF+ parent population are shown for blank and 12.5% PA standard; the Protein region was used to identify PA events that are ProteoStat® +



C. PA number concentrations for each standard plotted for BF and FL (ProteoStat®+) detection. PA concentrations measured on the ISX initially correlate with percent aggregated protein and then decrease. Note higher counts are measured for the blank sample using BF detection compared with FL detection.



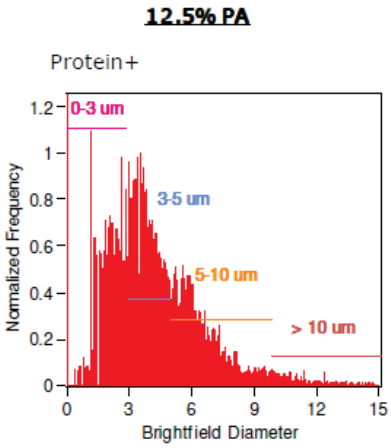
B. Representative images of ProteoStat® positive and negative events collected on ISX

Signal: Noise ratio

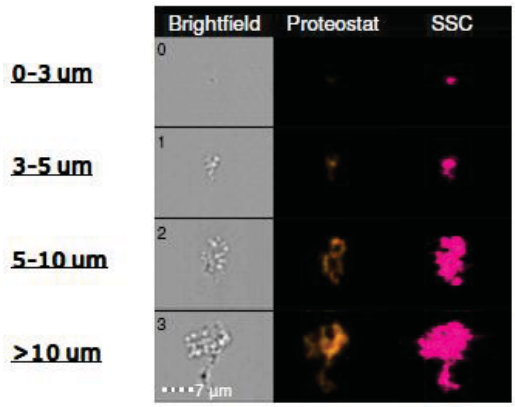
	Blank	0.0%	0.2%	0.4%	0.8%	1.6%	3.1%	6.3%	12.5%
Brightfield	1	1.4	1.9	3.7	3.0	4.4	5.3	3.8	4.1
Fluorescent	1	362	880	4165	3675	7249	9386	6200	7092

D. Signal-to-noise (S/N) ratios for each standard plotted for BF and FL detection. S/N calculated by dividing sample particle concentration by blank particle concentration. A lower background count for FL detection compared to BF results in significantly higher S/N.

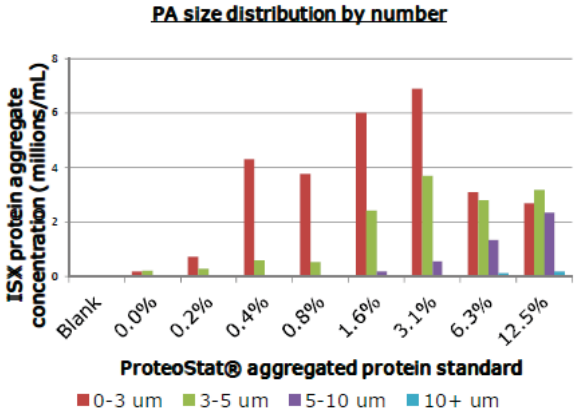
Figure 3: Analysis of protein aggregate size distribution

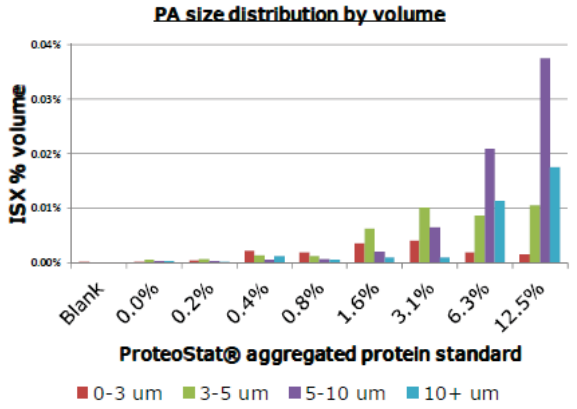


A. Histogram plotting BF diameter from the PA population; regions are used to identify PA in the 0-3, 3-5, 5-10, and 10-100 size range.

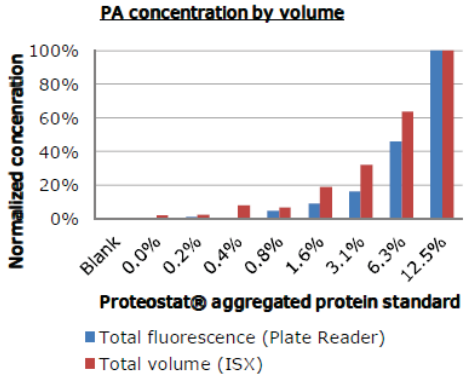


B. Representative images of PA events within each size range collected on ISX.



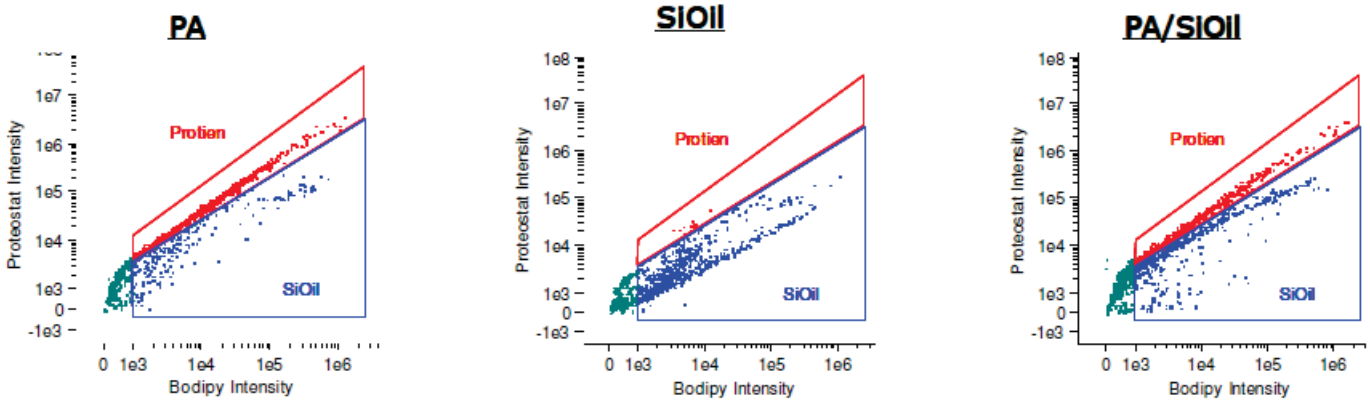


C. Number and volume distributions of 0-3, 3-5, 5-10, and 10-100 PA. Note that a higher fraction of larger aggregates (>5 um) is observed for samples with higher PA concentration, which has a significant impact on volume contribution of each size range.



D. Comparison of absolute concentration measurements of PA using ISX and FL plate reader. Values are normalized to one. Percent volume measured on the ISX correlates well with total fluorescence values measured on the plate reader.

Figure 4: Multiplexing detection of silicone oil microdroplets (SiOil) with BODIPY fluorescent dye

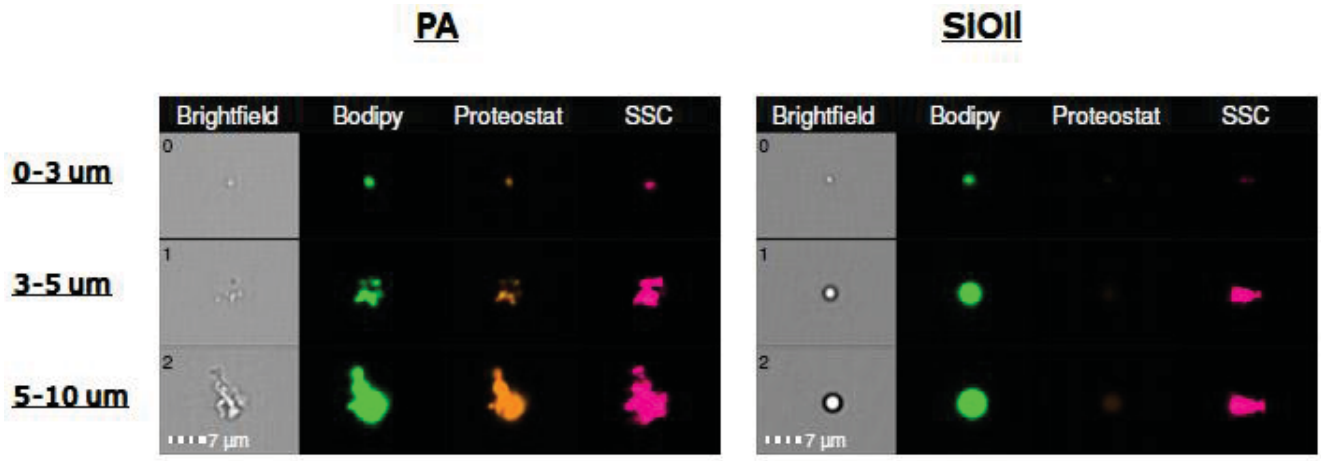


A. Bivariate intensity plots for Bodipy vs. ProteoStat® channels for samples containing SiOil, PA, and SiOil/PA. Regions were drawn to identify PA (Protein) and SiOil populations.

Brightfield image analysis

	Protein Aggregate			Silicone Oil		
Parameter						
Diameter	2.379	4.546	9.138	2.865	3.982	5.186
Circularity	7.112	3.172	4.29	8.827	12.7	14.56
Contrast	5.073	5.874	10.12	6.799	36.25	34.91

B. Comparison of BF parameters relevant for image based discrimination of PA and SiOil. Circularity is a measure of how much the object radius varies; round objects will have high circularity scores and irregular objects will have low circularity scores. Contrast measures the amount of variation in pixel intensity within an object; translucent objects will have low contrast scores and opaque objects will have high contrast scores. Note that BF parameters contrast and circularity increase with the size of the particle. Small PA and SiOil particles (0-3 um) have little difference in BF parameters but can still be discriminated with FL.



C. Representative images of SiOil and PA particles showing channels for Bodipy and Proteostat® FL collected on ISX

Conclusion

Particle characterization remains a critical challenge for the pharmaceutical industry due to a lack of suitable analytical techniques. In this study we evaluated the ability of the ImageStreamX® to overcome the limitations of current technologies. We found that the ProteoStat® Protein Aggregation Standards provided useful tool for evaluating the sensitivity and linearity of ISX for measuring PA, and the ProteoStat® Protein Aggregation assay enabled highly specific fluorescent staining of PA. Using the ISX we demonstrated sensitive detection of PA as low as 0.2%, and nominal counts were measured for the blank sample. Size analysis for PA at varying concentration provided a clear understanding of the relationship between particle counts, size distribution by number, and size distribution by volume. Furthermore, ability to multiplex particle classification was demonstrated using silicone oil microdroplets and a Bodipy fluorescence stain. In conclusion the ImageStreamX MKII imaging flow cytometer offers a number advantages compared to currently accepted industry standards for particle characterization, including (i.) direct particle size measurements in submicron to 100 micron range, (ii.) particle classification using fluorescence or image filtering, (iii.) quantitative measurement of potentially relevant morphological properties such as shape or texture, (iv.) absolute concentration measurements, and (v.) low sample volume requirement of 20 µL.

References

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